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A Thesis for the Degree of Doctor of Philosophy

Application of chlorine dioxide (ClO_2) gas treatment for inactivation of foodborne pathogens

식중독 균 제어를 위한 이산화염소 가스 처리의 적용

August, 2016

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Abstract

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Chlorine dioxide (ClO_2) has emerged as a promising non-thermal sanitizing technology. ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum. Its efficacy is largely not affected by pH and organic matter and it does no react with nitrogen compounds to form chloramines. The most widely accepted antimicrobial mechanism of ClO_2 is damage to protein synthesis and increased permeability of the outer cell membrane. ClO_2 gas may be more effective for inactivation of foodborne pathogens than aqueous ClO_2 due to its penetration ability. Also, ClO_2 gas could be applied for microbial control during transportation and storage of food. Several studies have been evaluated the antimicrobial effect of ClO_2 gas against foodborne pathogens on food and food contact surfaces. However, there is little information about factors affecting the antimicrobial efficacy of ClO_2 gas. Also, a few studies are available that evaluate the antimicrobial efficacy of the combination treatment of ClO_2 gas with other technology.

The specific objectives of this study were, (ⅰ) to investigate the effect of relative humidity, surface characteristics of samples, and temperature on the

antimicrobial efficacy of ClO_2 gas against *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* on produce and food contact surfaces, (ii) evaluate the antimicrobial effects of the combination treatment of ClO_2 gas with ultraviolet (UV) radiation, aerosolized sanitizer, and dry heat against foodborne pathogens on produce and seeds, (iii) develop portable sustained release formulation of ClO_2 gas for field application.

Spinach leaves and tomatoes were inoculated with three foodborne pathogens and treated with ClO_2 gas at different concentrations (1, 5, 10, 30, or 50 ppmv) for up to 20 min under differing conditions of RH (50, 70, and 90%). As ClO_2 gas concentration and treatment time increased, significant differences ($p < 0.05$) were observed between inactivation levels under different RH conditions. Generally, there were no significant differences ($p > 0.05$) in reduction levels of the three foodborne pathogens between 50 and 70% RH on spinach leaves. Exposure to 50 ppmv of ClO_2 gas for 20 min resulted in 1.25 to 1.78 (50% RH) and 2.02 to 2.54 (70% RH) log reductions of the three foodborne pathogens on spinach leaves. The levels of the three foodborne pathogens was reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 50 ppmv of ClO_2 gas under 90% RH. Exposure to 30 ppmv of ClO_2 gas (50% RH) for 20 min resulted in 1.22 to 1.52 log reductions of the three foodborne pathogens on tomatoes. Levels of the three foodborne pathogens were reduced to below the detection limit (0.48 log CFU/cm^2) within 15 min when exposed to 30 ppmv of ClO_2 gas at 70% RH, and within 10 min at 90% RH.

Treatment with 30 ppmv of ClO_2 gas did not significantly ($p > 0.05$) affect the color or texture of spinach leaves and tomatoes during 7 days of storage.

To evaluate the influence of surface properties of samples on the antimicrobial effect of ClO_2 gas against foodborne pathogens, the hydrophobicity of the selected surfaces was evaluated by water contact angle measurements. Also, white light scanning interferometry (WLSI) was used to acquire topographic images and surface roughness values of each surface. Produce (carrots, kale, cabbage, spinach, apples, tomatoes, and paprika) and food contact surfaces (Teflon, silicon, rubber, polyvinyl chloride, type 304 stainless steel with 2B or No.4 finish, and glass) inoculated with three foodborne pathogens were treated with 20 ppmv ClO_2 gas for up to 15 min. Contact angles of produce and food contact surfaces were highly and negatively correlated with the log reduction of all three pathogens. The R_a (arithmetic mean roughness) values of produce surfaces were negatively correlated with the log reductions of the three pathogens, although the correlation coefficients were quite lower than those between contact angle and the bacterial log reductions. The R_a values of food contact surfaces were not significantly ($p > 0.05$) correlated with the log reductions of the three pathogens. The results of this study showed that surface hydrophobicity is a more important factor relating to bacterial inactivation by ClO_2 gas from the surface than surface roughness.

Produce and food contact surfaces inoculated with three foodborne pathogens were treated with 20 ppmv ClO_2 gas at 15 and 25 ℃ under same conditions of

absolute humidity for up to 30 min to evaluate how treatment temperature influences the solubility of ClO_2 gas and the antimicrobial effect of ClO_2 gas. As treatment time increased, ClO_2 gas treatment at 15 ℃ caused significantly more ($p < 0.05$) inactivation of the three pathogens than ClO_2 gas treatment at 25 ℃. ClO_2 gas treatment at 15 ℃ for 30 min resulted in 0.99 to 1.65, 1.05 to 1.50, and 1.25 to 1.61 further log reductions of the three pathogens on spinach leaves, tomatoes, and stainless steel No.4, respectively, compared to 25 ℃ treatment. Treatment with ClO_2 gas at 25 ℃ for 20 min resulted in 1.88 to 2.31 log reductions of the three pathogens on glass while these pathogens were reduced to below the detection limit (0.48 log CFU/cm^2) within 15 min when treated with ClO_2 gas at 15 ℃. ClO_2 concentration on sample surfaces after ClO_2 gas treatment at 15 ℃ were significantly ($p < 0.05$) higher than those treated at 25 ℃.

The antimicrobial effect of the combined treatment of UV-C radiation (UVC) and ClO_2 gas against three foodborne pathogens on spinach leaves and tomato has been evaluated. In the case of spinach leaves, as treatment time increased the combined treatments of UVC and ClO_2 gas showed additive effects: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from the sum of individual treatments. On tomatoes, synergistic effects in inactivating *E. coli* O157:H7 and *S.* Typhimurium were observed after combination treatment of UVC and ClO_2 gas (10 ppmv) for 15 min or more. For both pathogens, inactivation achieved with the combination treatment was significantly ($p < 0.05$)

higher than the sum of UVC and ClO_2 gas (10 ppmv) inactivation. In the case of *L. monocytogenes*, the synergistic effect was observed after the combination treatment of UVC and ClO_2 gas (10 ppmv) for 20 min. Measuring leakage of UV-absorbing substances and analyzing transmission electron microscopy images provide evidence that damage to the cell membrane and changes to membrane permeability are involved in the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas. Combined treatment of UVC and ClO_2 gas (10 ppmv) did not significantly ($p > 0.05$) affect the color and texture of samples during 7 days of storage.

As an another available hurdle combination, the efficacy of ClO_2 gas combined with aerosolized sanitizer for decontaminating spinach leave and tomatoes was investigated. ClO_2 gas (5 or 10 ppmv) and aerosolized peracetic acid (PAA) (80 ppm) were applied alone or in combination for 20 min. Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 3.39, 3.29, and 3.36 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves, respectively. Treatment with 80 ppm of aerosolized PAA for 20 min caused 2.27, 1.89, and 0.84 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA (80 ppm) for 20 min caused 5.36, 5.06, and 4.06 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes experienced similar reduction patterns to those on spinach leaves. As treatment time increased, most combinations of ClO_2 gas and

aerosolized PAA showed additive effects in the inactivation of the three pathogens. Combined treatment of ClO_2 gas and aerosolized PAA produced injured cells of three pathogens on spinach leaves while generally did not produce injured cells of these pathogens on tomatoes. Combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA (80 ppm) did not significantly ($p > 0.05$) affect the color and texture of samples during 7 days of storage.

The antimicrobial effect of sequential treatment with ClO_2 gas and dry heat against foodborne pathogens on alfalfa and radish seeds was evaluated. Inoculated alfalfa and radish seeds were treated with 150 ppmv of ClO_2 gas for 1 h followed by 70 or 80 ℃ dry heat for 0, 1, 3 or 5 h. Dry heat treatment alone at 80 ℃ for 5 h resulted in 3.08 and 3.23 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, respectively. ClO_2 gas treatment alone for 1 h resulted in 1.22 to 1.45 and 1.58 to 1.61 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Subsequent dry heat treatment (80 ℃) for 5 h caused more than 5.32 and 5.29 log reduction of *E. coli* O157:H7 and *S.* Typhimurium, respectively. On radish seeds, dry heat treatment at 80 ℃ for 5 h resulted in 2.49 and 2.27 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, and sequential treatment with ClO_2 gas and dry heat (80 ℃) for 5 h caused 4.38 and 4.11 log reduction of *E. coli* O157:H7 and *S.* Typhimurium, respectively. The germination rate of seeds did not significantly decrease after sequential treatment except for radish seeds sequentially treated with ClO_2 gas and dry heat (80 ℃).

For on-site generation of ClO_2 gas without equipment, mixture composition for sustained release of ClO_2 gas was developed and its antimicrobial effect against foodborne pathogens on produce was evaluated. Sodium chlorite and citric acid were used to generate ClO_2 gas, and diatomaceous earth (DE) was used to induce sustained release of ClO_2 gas. Also, calcium chloride is used as a hydration accelerator. ClO_2 gas release profiles of various mixture compositions were observed under conditions of 50 and 90 % relative humidity (RH) for up to 36 h at 22 ± 1 ℃. RH affected the ClO_2 gas release profile, and the generation rate and maximum ClO_2 gas concentration could be controlled using DE and $CaCl_2$. When 9 and 12 g of DE were added to the mixture, ClO_2 gas concentration remained constant at 26 ± 1 ppmv for ca. 23 h and at 18 ± 1 ppmv for ca. 28 h, respectively, under conditions of 90 % RH. At 50% RH, when 0.05 g of $CaCl_2$ was added to mixtures containing 0.5 and 0.35 g of DE, ClO_2 gas concentration remained constant at 11 ± 1 ppmv for ca. 26 h and at 16 ± 1 ppmv for ca. 24 h, respectively. *E. coli* O157:H7 and *S.* Typhimurium were inoculated onto spinach leaves and tomatoes and exposed to ClO_2 gas at different concentrations (10, 20, or 30 ppmv) under 50 and 90% RH conditions for up to 20 min. More than 6.16 and 5.48, and more than 6.78 and 6.34 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes were observed after treatment with 30 ppmv of ClO_2 gas for 15 and 10 min, respectively, at 90% RH.

In conclusion, the results of this study are helpful for the food industry to establish ClO_2 gas treatment conditions for maximizing the antimicrobial efficacy of ClO_2 gas. The combination treatment of ClO_2 gas with other technology may suggest alternatives to currently used decontamination methods. Also, portable ClO_2 gas generating mixture could facilitate the use of ClO_2 gas in the food industry.

Keywords: chlorine dioxide gas, foodborne pathogen, inactivation, relative humidity, surface characteristics, temperature, ultraviolet, aerosolization, dry heat, sustained release

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Chapter I.

General introduction

I-1. Literature review

I-1.1. Properties of chlorine dioxide (ClO_2)

ClO_2 is a small, volatile, and a free radical while in aqueous solutions. It is neutral compound of chlorine in the +IV oxidation state. ClO_2 exists as a yellow-green gas at ambient temperature and the solution has a greenish yellow color when dissolved in water. It has chlorine-like odor that could be detected at 0.1 ppmv in air (Simpson, 2005). ClO_2 has high solubility in water, and it is approximately 10 times more soluble than chlorine (above 11 ℃). It does not hydrolyze in water and remains as dissolved gas form below 11 ℃ (Aieta and Berg, 1986). It is highly volatile and could be removed by aeration or agitation. A ClO_2 solution should keep in a sealed container under dark conditions, due to photolytic decomposition (White, 2010).

ClO_2 is a strong and selective oxidizing agent, and reacts by a one-electron transfer mechanism wherein it attacks electron rich centers in organic molecules and is reduced to ClO_2^- ion (Hoehn et al., 1996). It reacts by electrophilic abstraction and not by substitution reactions like chlorine. Consequently, it does not produce chlorinated organic byproducts such as trihalomethanes (THMs), and haloacetic acids (HAA). Also, ClO_2 does not react with ammonia or cause chloramines formation.

In neutral or near neutral solutions (pH 4-10), ClO_2 is stable in water while in highly alkaline (pH > 10) solutions, chlorate and chlorite ions are formed (EPA, 2006). ClO_2 completely dissociates into chloride ions due to the high available electrons at pH < 2 (Aieta, 1984).

ClO_2 is sensitive to physical shock, pressure, or temperature. It can decompose to chlorine and oxygen with heat and irradiation. It cannot be compressed without detonation, and compressed liquid ClO_2 is explosive at higher than 40 ℃. ClO_2 is explosive at concentrations over 10 % by volume in air or at a pressure higher than 76 mmHg (Simpson, 2005; White, 2010). Therefore, it can neither be stored nor transported and needs to be generate on-site prior to use.

Table I-1. Physical and chemical properties of ClO_2 (EPA, 2006).

Chemical formula	ClO_2
Chemical structure	O = Cl = O
Molecular weight	67.45 g/mol
Color	Gas phase: yellow green to orange Liquid phase: reddish-brown
Melting point	-59 ℃
Boiling point	11 ℃
Odor	Strongly pungent, chlorine-like
Physical state	Gas at room temperature
Vapor pressure	490 mmHg (0 ℃) ＞ 760 mmHg (25 ℃)
Stability	Unstable, estimated half in water ~25 min

I-1.2. Application of ClO_2

The use of ClO_2 as disinfectant dates back to the beginning of the 20th century, when it was used in Belgium for water disinfection (Tzanavaras et al., 2007). ClO_2 has been used to treat drinking water ever since the use of Cl_2 caused the formation of harmful by products such as THMs and haloacids. ClO_2 does not produce chlorination byproduct and it also seems to lower concentration of THMs precursors. Application of ClO_2 in drinking water has been for control of tastes and odors associated with algae and decaying vegetation. It is effective in control *Giardia*, *Crypotosporidium* viruses and bacteria which is advantageous for drinking water treatment (Gordon and Rosenblatt, 2005; White, 2010). Also, it is effective in destroying taste and odor producing phenolic compounds.

ClO_2 could be used to oxidize both iron and manganese. It reacts with iron and manganese to form precipitates which can be removed through sedimentation and filtration. ClO_2 reduces to chlorite ion in this reaction (Knocke et al., 1993). To remove 1.0 mg/L of iron and manganese, about 1.2 and 2.5 mg/L of ClO_2 is required, respectively.

For food processing application, Green Giant Co.'s Blue Earth plant in Minnesota was the first plant to retrofit with a ClO_2 treatment system (Kaur, 2013). The use of ClO_2 was effective in controlling growth of bacteria, biofouling in pea and corn canneries, and offensive odors were not produced in the plant. Since many

food processing plants have been used ClO_2 treatment system. ClO_2 is also effective for disinfection of poultry chiller water and has been approved for disinfection of fruits and vegetables. Also, it is used to disinfect manufacturing and laboratory equipment, environmental surfaces, tools, and clean rooms (EPA, 2006).

ClO_2 is also most commonly used in pulp bleaching. It produced strong bright white fibers without producing byproducts such as dioxins, furans or adsorbable organic halides (AOX) which were produce by chlorine treatment.

I-1.3. Regulation for use of ClO_2

The US EPA National Primary Drinking Water Regulations defines chlorite as a byproduct of drinking water treatment with a permitted maximum contaminant level (MCL) of 1.0 mg/L. The suggested no adverse response level (SNARL) is 0.38 mg/L by chronic exposure for a 70Kg human assuming 100% of the daily exposure is from 2L of drinking water, based on this recommended safety estimates. The National Secondary Drinking Water Regulations state that the maximum permitted level of chloride in water is 250 mg/L. With regards to daily intake, the World Health Organization (WHO) has established the tolerable daily intake values for chlorite and chlorate in drinking water as 0.03 mg/kg of body weight per day (WHO, 2005). Perchlorate has also been recently placed on the Drinking Water Candidate Contamination List and a primary drinking water regulation for perchlorate is being determined (EPA, 2011).

The US FDA Code of Federal Regulations (CFR) 173.300 permits the use of ClO_2 for food applications which is generated by a reaction between aqueous solution of sodium chlorite with either chlorine gas or a mixture of sodium hypochlorite and hydrochloric acid, treatment of an aqueous solution of sodium chlorate with hydrogen peroxide in presence of sulfuric acid or by treatment of an aqueous solution of sodium chlorite by electrolysis. ClO_2 is permitted as an antimicrobial agent in water used in poultry processing in an amount not to exceed 3

parts per million (ppm) residual ClO_2. Also, ClO_2 may be used as an antimicrobial agent in water used to wash fruits and vegetables that are not raw agricultural commodities in an amount not to exceed 3 ppm residual ClO_2. Treatment of the fruits and vegetables with ClO_2 shall be followed by a potable water rinse or by blanching, cooking, or canning. The US EPA approved the use of aqueous ClO_2 for fruit and vegetable washing, meat and poultry disinfection, and food processing equipment sanitation. Also, ClO_2 gas has been approved for use in manufacturing and for sanitizing laboratory equipment, environmental surfaces, tools, and clean rooms (EPA, 2015).

In case of ClO_2 gas, the Occupational Safety and Health Administration (OSHA) specifies the permissible exposure limit in air as 0.1 ppm (0.3 mg/m^3) and a short term exposure limit in air for 15 min as 0.3 ppm (0.9 mg/m^3). An exposure to ClO_2 exceeding the permissible exposure limits have been reported to be linked with respiratory and eyes irritation (NIOSH January 1992). The regulatory approval of ClO_2 gas for the surface decontamination of food is still pending despite the antimicrobial efficacy of ClO_2 gas. From the occupational point of view, application of ClO_2 gas in food industry need specialized worker safety programs, and closed systems for containment of concentrate leakage and fumes from volatilization (Suslow, 2000).

I-1.4. Antimicrobial effect of ClO_2

ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum (Aieta et al., 1984). The mechanism of inactivation by ClO_2 has been postulated by several studies. Oxidative attack on cell membrane proteins and enzyme and increased permeability of the outer cell membrane is the most widely accepted antimicrobial mechanism of ClO_2 (Aieta and Berg, 1986; Roller et al., 1980). ClO_2 reacts readily with the amino acids cysteine, methionine, tryptophan, and tyrosine causing damage to many proteins in the outer membrane, disrupting the ionic gradient, causing an increase in cell membrane permeability (Aieta and Berg, 1986). Also, it penetrates the cell membrane and damages proteins and enzymes within the cell that are necessary for protein synthesis (USDA, 2002).

The effects of different environmental factors on the antimicrobial activity of ClO_2 have been evaluated. Presence of suspended particle in the solution plays an important role in case of aqueous ClO_2 application. Protection from ClO_2 inactivation due to bentonite was determined to be approximately 11 percent for turbidities equal to or less than 5 NTUs and 25 percent for turbidities between 5 and 17 NTUs (Chen et al., 1984). Also, presence of organic matter results in faster degradation of ClO_2.

Temperature is also important factor for antimicrobial efficacy of aqueous ClO_2. Similar to chlorine, the effectiveness of ClO_2 decreases with decrease in temperature.

Aqueous ClO_2 was more effective at reducing *E. coli* O157:H7 and *Pseudomonas aeruginosa* at 20 ℃ than at 10 ℃ (Taylor et al., 1999). Increasing temperature within the range of 5-30 ℃ led to increased inactivation of *Mycobacterium avium* by aqueous ClO_2 (Vicuña-Reyes et al., 2008).

For aqueous ClO_2, pH of the solution affects the inactivation efficacy but to a lesser extent compared to chlorine. Antimicrobial efficacy of ClO_2 is not affected within the pH range of 6-10 (Dychdala, 1991). However, Bernarde et al. (1965) reported increase in pH from 6.5 to 8.5 increased the killing rate of *E.coli* by aqueous ClO_2. More research is needed to further understand the impact of pH on the antimicrobial efficacy of ClO_2.

In case of ClO_2 gas, Han et al. (2001a) evaluated the effects of ClO_2 gas concentration (0.1 to 0.5 mg/L), relative humidity (RH) (55 to 95%), treatment time (7 to 135 min), and temperature (5 to 25 ℃) on inactivation of *Escherichia coli* O157:H7 on green peppers using response surface methods. The order of significance from the most important to the least was: gas concentration, treatment time, RH, and temperature. Also, a synergistic effect of ClO_2 gas concentration and RH was observed. When green peppers were treated with 0.3 mg/L ClO_2 gas at 15 ℃, reductions increased from 1.93 to 4.00 log CFU/5g as RH increased from 55 to 95%. Growth of *Lactobacillus buchneri* on stainless steel strips after treatment with 8 mg/L ClO_2 gas for 10 min decreased as RH increased from 56 to 94% (Han et al., 1999). Woodworth and Jeng (1990) reported higher killing effect of ClO_2 gas

with humidification for *Bacillus subtilis* spores. Westphal et al. (2003) demonstrated that *Bacillus thuringiensis* spores swell with increasing RH. Swelling of a spore increases the diameter of channels for access of ClO_2 gas into spores and may account for the greater kill effectiveness of spores at high RH.

The amount of produce relative to the amount of ClO_2 gas applied is another important factor influencing the effectiveness of ClO_2 gas (Yuk et al., 2006). At a certain ClO_2 gas concentration, treatment chamber with a higher amount of sample will degrade the disinfectant faster than the case of a smaller amount of sample in a treatment chamber because the ClO_2 degradation rate is dependent on the amount of organic matter. Fast ClO_2 degradation could decrease the amount of gas available for disinfection during ClO_2 gas treatment.

Location of microorganism is other important factors. Han et al. (2000) found that ClO_2 gas treatment caused significantly more log reductions of *E. coli* O157:H7 on uninjured green bell pepper surfaces than on injured surfaces. Also, the inactivation of *Listeria monocytogenes* inoculated onto pulp skin of apple surfaces was more effective than these on calyx or stem cavities (Du et al., 2002).

I-1.4. Current studies on the antimicrobial effect of ClO_2 gas

The use of ClO_2 gas as disinfectant was first reported by Jeng and Woodworth (1990). They proved that ClO_2 gas can inactivate *Bacillus* species spores. In the 2000s, antimicrobial effect of ClO_2 gas has been evaluated against foodborne pathogens on produce surfaces. Also, the antimicrobial effect of ClO_2 gas against foodborne pathogens on several materials has been evaluated. Table I-2 and 3 show current studies on the antimicrobial effect of ClO_2 gas against pathogenic microorganisms on produce and food contact surfaces.

It is important to consider the actual ClO_2 gas concentration during treatment when comparing studies on inactivation of pathogen with ClO_2 gas. ClO_2 gas concentrations could increase, decrease, or keep constant depending on the type of ClO_2 gas generation and experimental setup.

Table I-2. Studies on the antimicrobial effect of ClO_2 gas against pathogenic microorganisms on produce.

Sample	Microorganism	ClO_2 concentration	Time (min)	RH (%)	Log reduction	Reference
Cabbage	*Salmonella*		30.8		4.42	Sy et al. (2005a)
	E. coli O157:H7	4.1 mg/L	20.5	48-85	3.13	
	L. monocytogenes		29.3		3.60	
Carrot	*Salmonella*		30.8		5.15	
	E. coli O157:H7	4.1 mg/L	20.5	51-88	5.62	
	L. monocytogenes		29.3		5.88	
Lettuce	*Salmonella*		30.8		1.58	
	E. coli O157:H7	4.1 mg/L	20.5	36-84	1.57	
	L. monocytogenes		29.3		1.53	
Apple	*Salmonella*	4.1 mg/L	25	35-68	4.21	
Tomatoes	*Salmonella*	4.1 mg/L	25	34-62	4.33	
Onion	*Salmonella*	4.1 mg/L	25	35-64	1.94	
Peach	*Salmonella*	4.1 mg/L	25	55-78	3.23	
Blueberry	*Salmonella*	4.1 mg/L	30	76-90	2.95	Sy et al. (2005b)
Strawberry	*Salmonella*	4.1 mg/L	30	85-88	2.32	
Raspberry	*Salmonella*	4.1 mg/L	30	75-83	0.52	
Blueberry	*E. coli* O157:H7				4.25	Popa et al. (2007)
	Salmonella	4 mg/L	720	99.9	3.62	
	L. monocytogenes				3.94	
Mungbean sprout	*Salmonella*	0.5 mg/L	60	50-75	5.5	Prodduk et al. (2014)

Table I-2. (continued).

Sample	Microorganism	ClO_2 concentration	Time (min)	RH (%)	Log reduction	Reference
Green bell pepper	*E. coli* O157:H7	1.2 mg/L	30	90-95	6.45	Han et al. (2000a)
	E. coli O157:H7	1.2 mg/L	30	90-95	> 8.04	Han et al. (2000b)
	L. monocytogenes	3.0 mg/L	10	90-95	> 6	Han et al. (2001)
Apple	*L. monocytogenes*	4.0 mg/L	30	90	> 5.5	Du et al. (2002)
	E. coli O157:H7	7.2 mg/L	10	90-95	> 5.8	Du et al. (2003)
Strawberry	*E. coli* O157:H7				4.6	Mahmoud et al.
	Salmonella enterica	5.0 mg/L	10	90-95	4.3	(2007)
	L. monocytogenes				4.7	
	E. coli O157:H7	3.0 mg/L	10	90-95	> 5.0	Han et al. (2004)
	L. monocytogenes				> 5.0	
	E. coli O157:H7				2.7	Trinetta et al.
	Salmonella	10.0 mg/L	3	90-95	4.8	(2013)
	L. monocytogenes				3	
Lettuce	*E. coli* O157:H7	5.0 mg/L	10	90-95	4.6	Mahmud and
	S. enterica				4.3	Linton (2008)
Cantaloupe	*E. coli* O157:H7		10		4.6	Mahmoud et al.
	Salmonella Poona	5.0 mg/L	6	90-95	5	(2008)
	L. monocytogenes		10		4.3	
	E. coli O157:H7				2.0	Trinetta et al.
	Salmonella	10.0 mg/L	3	90-95	4	(2013)
	L. monocytogenes				3.3	

Table I-2. (continued).

Sample	Microorganism	ClO_2 concentration	Time (min)	RH (%)	Log reduction	Reference
Spinach	*E. coli* O157:H7	2.1 mg/L	60	-	0.7	Neal et al. (2012)
	Salmonella				0.6	
Tomatoes	*S. enterica*	0.5 mg/L	10	90	> 5.0	Bhagat et al. (2010)
	L. monocytogenes				> 5.0	
	Salmonella	2 mg/L	3	90-95	2.41	Trinetta et al. (2010)
		5 mg/L			2.43	
		8 mg/L			3.77	
		10 mg/L			4.78	
Orange	*S. enterica*	0.5 mg/L	10	90	> 5.0	Bhagat et al. (2011)

Table I-3. Studies on the antimicrobial effect of ClO_2 gas against pathogenic microorganisms on food contact surfaces.

Surfaces	Microorganism	ClO_2 concentration	Time (min)	RH (%)	Log reduction	Reference
Stainless steel	*L. monocytogenes* Biofilm	0.3 mg/L	10	75	3.2	Vaid et al. (2010)
	L. monocytogenes Biofilm	1.0 mg/L 2.0 mg/L	10	75	2.5 3.8	Trinetta et al. (2012)
Paper	*B. thuringiensis* spore	10.0 mg/L	720	85-92	3.6	Han et al. (2003)
Wood					5.0	
Epoxy					5.7	
Plastic					5.8	
Stainless steel	*B. subtilis* spore	0.08 % (v/v)	180	75	ca. 4.6	Li et al. (2012)
Painted steel					ca. 5.9	
Polyvinyl chloride					ca. 6.5	
Polyurethane					ca. 4.7	
Glass					ca.5.0	
Cotton cloth					ca. 1.5	
Glass	*E. coli*	0.05 ppmv	180	54	>2.0	Morino et al. (2011)
	S. aureus		300		>2.0	

I-2. Limitation of current studies on ClO_2 gas

Most studies just evaluated the antimicrobial effect of ClO_2 gas against several pathogens on various produce and food contact surfaces. Based on the literature review of current studies on the antimicrobial effect of ClO_2 gas, some limitations are identified. First of all, there was only one study that evaluated factors affecting antimicrobial effect of ClO_2 gas (Han et al., 2001a). However, this study used batch type ClO_2 gas treatment system, so it seems that it was difficult to get an accurate result. At this point, more research is needed to evaluate factors affecting antimicrobial effect of ClO_2 gas. The specific needs are as follow:

(ⅰ) Most studies have evaluated the antimicrobial effect of ClO_2 gas under conditions of high RH (> 80%) because it is well known that the antimicrobial effect of ClO_2 gas increases with increasing RH. However, there have been no studies to evaluate the inactivation tendency of ClO_2 gas according to levels of RH and treatment time. This may be an important factor for practical application of ClO_2 gas by the food processing industry.

(ⅱ) There are no studies considering the influence of surface properties of produce and food contact surfaces on the inactivation efficacy of ClO_2 gas. Also, comparative data for different produce and food contact surfaces subjected to the same treatment are not readily available. Surface properties such as surface

hydrophobicity and topography could influence bacterial inactivation from a surface (Wang et al., 2009).

(iii) There have been no studies considering the influence of treatment temperature on the inactivation efficacy of ClO_2 gas. Although Han et al. (2001a) studied the effects and interactions of temperature (5 to 25 ℃) and RH (55 to 95%), absolute humidity (AH) should be used to compare the effect of different treatment temperatures on the solubility of ClO_2 gas. Treatment temperature could be an important factor affecting antimicrobial efficacy of ClO_2 gas since it could affect ClO_2 gas solubility.

Secondly, the concentration of ClO_2 gas used in previous studies was excessive (about 180-3600 ppmv). These concentrations were much higher than a LC50 value (32 ppmv, 90 mg/m^3) determined for rats as a single exposure (Dobson, 2002). Combinations of different technologies, known as hurdle technology, could be an alternative to the use of high ClO_2 gas concentrations. Combined treatments can achieve required levels of food safety and the maintenance of organoleptic qualities of foods, while decreasing the intensity of each hurdle, that is, the antimicrobial concentration (Leistner and Gorris, 1995). In case of aqueous ClO_2, combined treatments of aqueous ClO_2 with other methods such as UV-C (Kim et al., 2009a), dry heat (Kim et al., 2010a), ultrasound (Chen and Zhu, 2011), modified atmosphere packaging (Jin and Lee, 2007), and sanitizers (Kim et al., 2009b; Pohlman et al.,

2002), have been studies. However, there are no studies on the efficacy of combination of ClO_2 gas and other methods in inactivation foodborne pathogens, except dry heat (Annous and Burke, 2015).

Thirdly, there are some factors limiting widespread use of ClO_2 gas in disinfection activities in spite of its antimicrobial effect and several advantages over aqueous sanitizers (Stubblefield and Newsome, 2015). ClO_2 gas should be prepared at the application site due to its instability in shipment. Also, generation of ClO_2 gas typically entails the use of complicated equipment and trained personnel (Stubblefield and Newsome, 2015). Although several methods for on-site generation of ClO_2 gas without equipment have been developed (Fred, 2002; Isaac and Tenney, 2014; Yang and Kim, 2005; Engelhard Corporation, 2001), there are no suitable products for food application. ClO_2 gas sachets have been applied for the inactivation of foodborne pathogens on foods (Sy et al., 2005; Popa et al., 2007; Wu et al, 2010). However, these sachets release too much ClO_2 gas in a short time and it is difficult to control the generation rate of ClO_2 gas.

I-3. Objectives of this study

The specific objectives of this study were,

(ⅰ) to investigate intrinsic and extrinsic factors affecting the antimicrobial effect of ClO_2 gas against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on produce and food contact surfaces.

The effect of varying levels of RH on the antimicrobial effect of ClO_2 gas on spinach leaves and tomatoes, and the quantity of solubilized ClO_2 gas on those surfaces was measured after exposure to ClO_2 gas under different RH conditions to determine how they affect inactivation patterns of pathogens. Also, this study examined how surface properties (hydrophobicity and roughness) of produce and food contact surfaces influence the antimicrobial effect of ClO_2 gas against foodborne pathogens on these selected surfaces. Lastly, this study determined how ClO_2 gas treatment temperature influences the antimicrobial effect of ClO_2 gas against foodborne pathogens on produce and food contact surfaces. To assess the influence of treatment temperature on the solubility of ClO_2 gas, ClO_2 concentration on sample surfaces were analyzed.

(ⅱ) to evaluate the antimicrobial effect of the combination treatment of ClO_2 gas with ultraviolet (UV) radiation, aerosolized sanitizer, and dry heat against foodborne pathogens on produce and seeds.

(iii) to develop a mixture composition which could constantly release a low concentration of ClO_2 gas suitable for field application. Also, the antimicrobial effect of ClO_2 gas generated by this mixture against foodborne pathogens on spinach leaves and tomatoes was evaluated and compared to ClO_2 gas generated by a lab scale ClO_2 gas generating system.

Chapter II.

Extrinsic and intrinsic factors affecting antimicrobial effect of ClO_2 gas against foodborne pathogens

II-1. Effect of relative humidity on the antimicrobial effect of ClO_2 gas against foodborne pathogens on fresh produce

II-1.1. Introduction

Foodborne outbreaks related to the consumption of fresh and fresh-cut produce have increased in recent years (FDA, 2011). From 1996 to 2008, about 82 foodborne illness outbreaks were related to the consumption of fresh produce, and among these outbreaks 28 cases were associated with leafy greens, accounting for 949 illnesses and 5 deaths (FDA, 2009). In 2006, fresh spinach and spinach-containing products were associated with an outbreak of *Escherichia coli* O157:H7 (CDC, 2006; Maki, 2006). Most of the 204 cases and 3 deaths occurred in the United States, and one case occurred in Canada (Jay et al., 2007; Wendel et al., 2009). Also, a total of 33 persons infected with *E. coli* O157:H7 traced to organic spinach and spring mix blend was reported from 5 US states in 2012 (CDC, 2012). In case of tomatoes, human salmonellosis comprising 5,324 cases, including 35 outbreaks, were documented in the United States from 1990 to 2012 (Wang and Ryser, 2014). Especially, various *Salmonella* enterica serotypes have been implicated in several large multi-state episodes with hundreds of clinical cases (CDC, 2005, 2007).

ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum (Trinetta et al, 2012). The mechanism of disinfection by ClO_2 has been postulated by several studies. The most widely accepted antimicrobial mechanism of ClO_2 is damage to protein synthesis and increased permeability of the outer cell membrane (Aieta and Berg, 1986; Roller et al., 1980). Efficacy of ClO_2 is largely not affected by pH and

organic matter and it does no react with nitrogen compounds to form chloramines (Aieta et al., 1984; Beuchat, 1998).

ClO_2 gas may be more effective for inactivation of foodborne pathogens than aqueous ClO_2 due to its penetration ability (Han et al., 2001b). Also, ClO_2 gas could be applied for microbial control during transportation and storage of fresh produce. Antimicrobial effect of ClO_2 gas has been evaluated against foodborne pathogens on fresh produce such as apples (Du et al., 2002), green peppers (Han et al., 2000), lettuce (Mahmoud and Linton, 2008; Sy et al., 2005), cabbage (Sy et al., 2005), carrots (Gómez-López et al., 2007), tomatoes (Bhagat et al., 2010; Trinetta et al., 2013), blueberries (Popa et al., 2007; Sy et al., 2005), and strawberries (Han et al., 2004).

Gas concentration, relative humidity (RH), treatment time, and temperature can affect the antimicrobial effect of ClO_2 gas, and especially, the combination of gas concentration and RH shows a synergistic effect (Han et al., 2001a). Several studies have been conducted under conditions of high RH (>80%) to evaluate the antimicrobial effect of ClO_2 gas (Bhagat et al., 2010; Bhagat et al., 2011; Gómez-López et al., 2008; Popa et al., 2007; Vandekinderen et al., 2009), but there have been no studies to evaluate the inactivation tendency of ClO_2 gas relative to conditions of RH and treatment time.

This study evaluated the effect of varying levels of RH on the antimicrobial effect of ClO_2 gas against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria*

monocytogenes on spinach leaves and tomatoes. Also, the quantity of solubilized ClO_2 gas on spinach leaves and tomato surfaces was measured after exposure to ClO_2 gas under different RH conditions to determine how they affect inactivation patterns of pathogens. After treatment, any changes in color and texture of spinach leaves and tomatoes were assessed.

II-1.2. Materials and Methods

Bacterial strains. Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104), and *L. monocytogenes* (ATCC 19111, ATCC 19115, and ATCC 15313) were obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea).

Culture preparation and sample inoculation. Each strain (maintained on-80 ℃ frozen stocks) was streaked for isolation onto tryptic soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) and incubated at 37 ℃ for 24 h. A single colony of each strain was inoculated into 5 ml of tryptic soy broth (TSB; Difco), incubated at 37 ℃ for 24 h, collected by centrifugation at 4000 × g for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7–10^8 CFU/ml. Afterwards, suspended pellets of the three pathogens were combined to comprise a mixed culture cocktail containing approximately equal numbers of cells of each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*.

Spinach and tomatoes were purchased from a local market (Seoul, South Korea) on the day of experiments. Spinach and tomatoes were washed in running water and dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h before experiments. Spinach leaves were trimmed to approximately 5 × 3 cm in size. The outer surface

of tomatoes was cut into 5 × 2 cm pieces. Prepared spinach leaves and tomatoes were placed on aluminum foil in a laminar flow biosafety hood, and 0.1 ml of culture cocktail was inoculated onto one side of the sample by depositing droplets with a micropipettor at 15–20 locations. After inoculation, samples were dried in a laminar flow biosafety hood for 1 h at 22 ± 2 ℃ with the fan running.

ClO_2 gas treatment system. Fig. II-1 shows ClO_2 gas treatment system used in this experiment. ClO_2 gas was prepared using a ClO_2 gas generating system (Daehan E&B, Goyang-si, South Korea). Generated ClO_2 gas was introduced into the polyvinyl chloride treatment chamber (length × width × height, 0.7 × 0.5 × 0.6 m), and continuously circulated using a ring blower (HRB-101, Hwanghae electronic, Incheon, South Korea). The concentration of ClO_2 gas in the treatment chamber was continuously monitored and controlled using a ClO_2 gas transmitter (ATi F12, Analytical Technology, U.K.). A humidifier (H-C976, Osungsa, Changwon-si, South Korea) was used to control RH in the treatment chamber. A thermohygrometer (YTH-600, Uins, Seoul, South Korea) was used to measure RH and temperature in the treatment chamber.

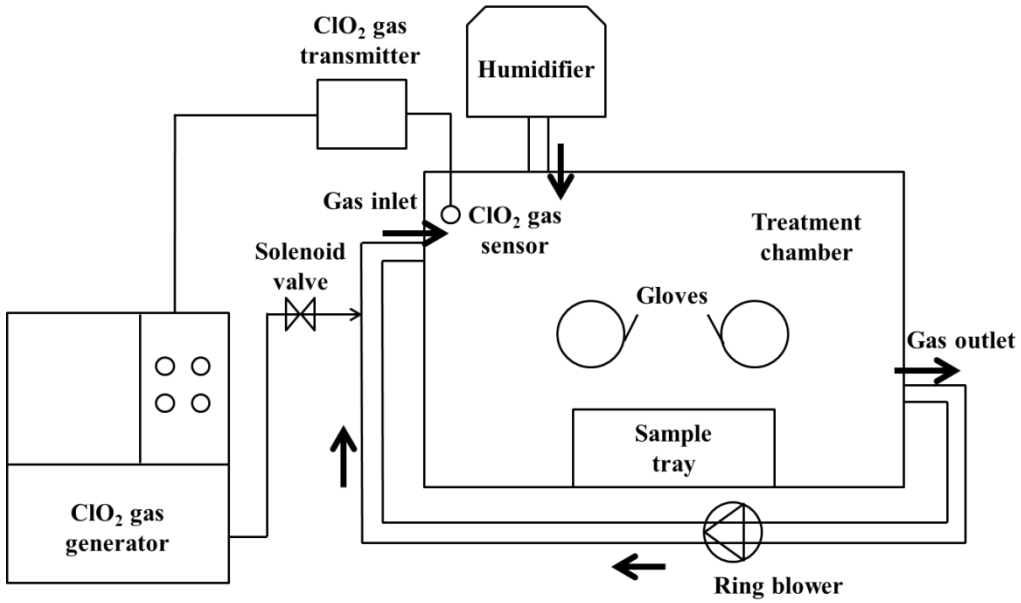


Fig. II-1. Schematic diagram of the ClO_2 gas treatment system used in this experiment.

ClO_2 gas treatment. Inoculated spinach leaves and tomatoes were placed on sterile plastic sample tray inside the treatment chamber with the inoculated surfaces facing upwards and covered with a plastic lid. Spinach leaves were treated with 1, 5, 10, 30, and 50 ppmv ClO_2 gas for 20 min, and tomatoes were treated with 1, 5, 10, 20, and 30 ppmv ClO_2 for 20 min at 22 ± 2 ℃, respectively. The RH of the treatment chamber was adjusted to 50, 70, and 90% with an accuracy of ± 2%. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and the inoculated side of spinach leaves and tomatoes was exposed to ClO_2 gas. Samples were withdrawn from the treatment chamber after 1, 5, 10, 15, and 20 min exposure to ClO_2 gas, and treated samples were used to determine surviving bacterial populations. These experiments were repeated three times.

Bacterial enumeration. Treated spinach leaves (10 ± 0.2 g) and one piece of tomatoes were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 and 30 ml of neutralizing buffer (Difco), respectively, Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. After homogenization, 1 ml aliquots withdrawn from stomacher bags were tenfold serially diluted in BPW, and 0.1 ml of appropriate diluents were spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L.*

monocytogenes, respectively. Where low levels of surviving cells were expected, 1 ml aliquots withdrawn from stomacher bags were divided between four plates of each medium and spread-plated to lower the detection limit. The plates were incubated at 37 ℃ for 24–48 h. Colonies were counted after incubation and calculated as log CFU/g for spinach leaves and log CFU/cm^2 for tomatoes.

ClO_2 concentration on sample surfaces after treatment. Spinach leaves were exposed to 10 and 30 ppmv of ClO_2 gas under different RH conditions (50, 70, and 90%) for 20 and 15 min, respectively. Whole tomatoes were exposed to 10, 20, and 30 ppmv of ClO_2 gas under different RH conditions (50, 70, and 90%) for 20, 15, and 10 min, respectively. After treatment, spinach leaves (10 ± 0.2 g) and tomatoes were immediately rinsed with 100 ml of sterile distilled water in sterile stomacher bags and massaged by hand for 5 min. Ten milliliters of sample were removed from each stomacher bag and tested by the DPD (N, N-diethyl-p-phenylenediamine) method using a Hach DR/820 Colorimeter (Hach, Loveland, CO) (Trinetta et al., 2011). The limit of detection for this method is 0.04 mg/L. ClO_2 concentration in rinse water were reported as mg/L and subsequently converted to mg/kg of produce. Untreated spinach leaves and tomatoes were used as a control. These experiments were conducted in triplicate.

Color and texture measurement. Treated and untreated (control) spinach leaves and whole tomatoes (uninoculated) were stored in Ziploc® bag at 4 ℃ (spinach leaves) and 12 ℃ (tomatoes) for 7 days to identify changes in quality

during storage following treatments. Color values (Hunter's L, a, b) of spinach leaves and tomatoes were measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each sample. The texture of spinach leaves and tomatoes was evaluated with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set and cylinder probe with a 4 mm diameter, respectively. Twenty grams of spinach leaves was placed onto the press holder with the stems positioned perpendicular to the path of the blade, and a blade was moved down at 2 mm/s (path length 10 mm). For tomatoes, the loading rate and path length were set at 2 mm/s and 10 mm. Three measurements were performed with independently-prepared samples for each treatment. Texturepro CT software (Brookfield Engineering Laboratories, Inc.) was used to record maximum force.

Statistical analysis. All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC, USA) and discrimination of means by Duncan's multiple range test at a probability level of $p < 0.05$.

II-1.3. Results

Effects of ClO_2 gas treatment on populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves. Table II-1 to 5 show the effects of ClO_2 gas treatment against the three pathogens on spinach leaves. Population before treatment was ca. 6–7 log CFU/g for *E. coli* O157:H7 and *S.* Typhimurium, and ca. 5–6 log CFU/g for *L. monocytogenes*. When spinach leaves were treated with 1 ppmv ClO_2 gas, reduction levels of the three pathogens were not significantly ($p > 0.05$) different between varying levels of RH (Table II-1). After treatments under 50, 70, and 90% RH for 20 min, 0.83, 1.01, and 1.12 log reductions of *E. coli* O157:H7 were achieved, respectively. *S.* Typhimurium was reduced by 0.82, 0.90, and 1.22 log after 20 min treatment under 50, 70, and 90% RH, respectively. One ppmv ClO_2 gas treatment under 50, 70, and 90% RH for 20 min resulted in 0.57, 0.68, and 1.02 log reductions of *L. monocytogenes*, respectively.

As ClO_2 gas concentration increased, treatment under 90% RH resulted in more significant differences ($p < 0.05$) in reduction levels of the three foodborne pathogens than ClO_2 gas treatment under 50 and 70% RH. Generally, there were no significant differences ($p > 0.05$) in reduction levels of three foodborne pathogens between treatments of 50 and 70% RH. Treatment with 5 ppmv of ClO_2 gas for 20 min caused 0.84 to 1.34 (50% RH), 1.32 to 1.41 (70% RH), and 1.94 to 2.36 (90% RH) log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*

(Table II-2). Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 1.20 to 1.32 (50% RH), 1.53 to 2.09 (70% RH), and 3.56 to 3.63 (90% RH) log reductions of the three foodborne pathogens (Table II-3). Treatment with 30 ppmv of ClO_2 gas for 20 min caused 1.25 to 1.54 (50% RH) and 1.55 to 1.76 (70% RH) log reductions of the three foodborne pathogens (Table II-4). The numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 30 ppmv of ClO_2 gas under 90% RH. Exposure to 50 ppmv of ClO_2 gas for 20 min resulted in 1.25 to 1.78 (50% RH) and 2.02 to 2.54 (70% RH) log reductions of the three foodborne pathogens (Table II-5). The levels of the three foodborne pathogens were reduced to below the detection limit (1 log CFU/g) within 15 min when treated with 50 ppmv of ClO_2 gas under 90% RH.

Table II-1. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 1 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.15(0.28)Aa[b]	0.53(0.17)ABa	0.58(0.16)ABa	0.75(0.33)Ba	0.83(0.08)Ba
	70	0.37(0.03)Aa	0.63(0.15)ABa	0.75(0.08)BCa	0.88(0.30)BCa	1.01(0.02)Ca
	90	0.30(0.22)Aa	0.52(0.35)ABa	0.69(0.51)ABa	0.89(0.22)ABa	1.12(0.39)Ba
S. Typhimurium	50	0.20(0.29)Aa	0.22(0.30)Aa	0.35(0.39)Aa	0.47(0.64)Aa	0.82(0.04)Aa
	70	0.25(0.18)Aa	0.41(0.24)Aa	0.49(0.05)Aa	0.59(0.17)ABa	0.90(0.27)Ba
	90	0.26(0.09)Aa	0.44(0.36)ABa	0.69(0.49)ABCa	0.94(0.26)BCa	1.22(0.29)Ca
L. monocytogenes	50	0.20(0.23)Aa	0.38(0.04)ABa	0.46(0.05)ABa	0.56(0.14)Ba	0.57(0.23)Ba
	70	0.10(0.15)Aa	0.32(0.25)Aa	0.61(0.50)Aa	0.64(0.56)Aa	0.68(0.50)Aa
	90	0.22(0.22)Aa	0.53(0.12)ABa	0.68(0.39)ABCa	0.80(0.30)BCa	1.02(0.05)Ca

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-2. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves after 5 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.46(0.70)Aa[b]	0.55(0.70)Aa	0.96(0.36)Aa	1.19(0.41)Aa	1.34(0.47)Aa
	70	0.60(0.32)Aa	0.89(0.41)ABa	1.12(0.50)ABa	1.33(0.18)ABa	1.40(0.48)Ba
	90	0.67(0.24)Aa	1.11(0.40)ABa	1.68(0.42)BCa	2.01(0.03)CDb	2.36(0.46)Db
S. Typhimurium	50	0.46(0.10)Aa	0.51(0.04)Aa	0.57(0.05)Aa	0.78(0.06)Ba	1.06(0.13)Ca
	70	0.69(0.39)Aa	0.86(0.55)Aa	1.03(0.27)Aa	1.29(0.37)Aab	1.41(0.24)Ab
	90	0.55(0.16)Aa	1.01(0.18)Aa	1.72(0.35)Bb	1.83(0.52)Bb	2.33(0.31)Bb
L. monocytogenes	50	0.38(0.31)Aa	0.51(0.11)ABa	0.56(0.07)ABa	0.56(0.15)ABa	0.84(0.16)Ba
	70	0.57(0.06)Aa	0.70(0.11)ABa	0.97(0.13)BCb	1.21(0.31)CDb	1.32(0.12)Db
	90	0.38(0.24)Aa	0.56(0.13)Aa	1.12(0.21)Bb	1.41(0.15)Bb	1.94(0.15)Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-3. Log reductions[a] of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on spinach leaves after 10 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.39(0.09)Aa[b]	0.50(0.21)Aa	0.91(0.13)Ba	1.14(0.31)BCa	1.32(0.22)Ca
	70	0.75(0.33)Aa	0.93(0.07)Ab	1.56(0.16)Bb	1.77(0.14)BCb	2.09(0.40)Cb
	90	0.85(0.25)Aa	1.22(0.15)Ab	2.51(0.32)Bc	3.08(0.41)Cc	3.63(0.37)Cc
S. Typhimurium	50	0.40(0.12)Aa	0.57(0.52)Aa	0.67(0.18)ABa	0.76(0.19)ABa	1.20(0.21)Ba
	70	0.76(0.04)Aa	0.97(0.14)ABa	1.21(0.13)Bb	1.23(0.14)Bb	1.68(0.24)Ca
	90	0.72(0.54)Aa	1.08(0.54)Aa	2.32(0.20)Bc	2.94(0.23)BCc	3.56(0.51)Cb
L. monocytogenes	50	0.41(0.05)Aa	0.39(0.42)Aa	0.49(0.05)Aa	0.91(0.42)ABa	1.21(0.39)Ba
	70	0.45(0.20)Aa	0.76(0.33)ABa	0.90(0.54)ABCa	1.21(0.46)BCa	1.53(0.28)Ca
	90	0.41(0.02)Aa	0.71(0.27)Ba	1.76(0.16)Bb	2.54(0.30)Cb	3.38(0.29)Db

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-4. Log reductions[a] of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on spinach leaves after 30 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.65(0.36)Aa[b]	0.82(0.16)ABa	1.12(0.34)ABCa	1.30(0.37)BCa	1.54(0.13)Ca
	70	0.73(0.15)Aa	0.78(0.22)ABa	1.13(0.32)Ba	1.53(0.09)Ca	1.70(0.19)Ca
	90	1.06(0.43)Aa	1.99(0.37)Bb	4.02(0.50)Cb	> 5.78Db	> 5.78Db
S. Typhimurium	50	0.50(0.08)Aa	0.64(0.11)ABa	0.88(0.10)BCa	1.07(0.29)CDa	1.25(0.10)Da
	70	0.64(0.19)Aa	0.88(0.21)Aa	1.16(0.03)Ba	1.42(0.03)Ca	1.76(0.11)Db
	90	0.98(0.10)Ab	1.69(0.08)Bb	3.76(0.32)Cb	> 5.68Db	> 5.68Dc
L. monocytogenes	50	0.71(0.25)Aa	1.07(0.40)ABa	1.12(0.13)ABa	1.17(0.19)ABa	1.45(0.18)Ba
	70	0.86(0.19)Aa	1.05(0.13)ABa	1.16(0.01)BCa	1.30(0.04)Ca	1.55(0.07)Da
	90	0.62(0.17)Aa	1.33(0.06)Ba	2.76(0.07)Cb	> 4.86Db	> 4.86Db

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-5. Log reductions[a] of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on spinach leaves after 50 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.74(0.15)Aa[b]	1.10(0.21)Ba	1.31(0.15)BCa	1.50(0.17)Ca	1.58(0.02)Ca
	70	0.50(0.16)Aa	0.82(0.28)Aa	1.44(0.16)Ba	1.80(0.26)Ba	2.45(0.35)Cb
	90	0.77(0.20)Aa	2.47(0.10)Bb	5.08(0.20)Cb	> 5.53Db	> 5.53Dc
S. Typhimurium	50	0.66(0.33)Aa	0.92(0.09)ABa	1.08(0.26)Ca	1.21(0.09)Ca	1.25(0.04)Ca
	70	0.60(0.15)Aa	0.73(0.07)ABa	1.30(0.23)BCa	1.49(0.11)Ca	2.54(0.65)Db
	90	1.09(0.32)Aa	2.34(0.59)Bb	5.19(0.23)Cb	> 5.56Db	> 5.56Dc
L. monocytogenes	50	0.64(0.08)Aa	1.26(0.41)Ba	1.31(0.03)Ba	1.55(0.27)BCa	1.78(0.09)Ca
	70	0.78(0.25)Aa	1.28(0.08)Ba	1.42(0.07)Ba	1.89(0.14)Ca	2.02(0.35)Ca
	90	0.76(0.23)Aa	1.98(0.24)Bb	4.71(0.18)Cb	> 4.85Db	> 4.85Db

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Effects of ClO_2 gas treatment on populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes. The effects of ClO_2 gas treatment against the three pathogens on tomatoes are shown in Tables II-6 to 10. After exposure to 1 ppmv of ClO_2 gas, reduction levels of the three pathogens did not significantly ($p > 0.05$) differ according to RH except for *E. coli* O157:H7 treated for 15 min or more (Tables II-6). As ClO_2 gas concentration increased, significant differences ($p < 0.05$) were observed between inactivation levels under different RH conditions. ClO_2 gas treatment at 90% RH caused significantly more ($p < 0.05$) inactivation of the three pathogens than ClO_2 gas treatment under conditions of 50 and 70% RH. Exposure to 5 ppmv of ClO_2 gas for 20 min caused 0.97 to 1.17 (50% RH), 1.54 to 1.81 (70% RH), and 2.14 to 2.37 (90% RH) log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively (Tables II-7). Treatment with 10 ppmv of ClO_2 gas for 20 min resulted in 1.10 to 1.33 (50% RH), 1.46 to 1.97 (70% RH), and 3.30 to 4.33 (90% RH) log reductions of the three foodborne pathogens (Tables II-8). Treatment with 20 ppmv of ClO_2 gas for 20 min caused 1.07 to 1.35 (50% RH) and 3.06 to 4.39 (70% RH) log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* (Tables II-9). The levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were reduced to below the detection limit (0.48 log CFU/cm^2) within 15 min when treated with 20 ppmv of ClO_2 gas at 90% RH. Treatment with 30 ppmv of ClO_2 gas for 20 min resulted in 1.22 to 1.52 (50% RH) log reductions of the three foodborne pathogens (Tables II-

10). Levels of the three foodborne pathogens were reduced to below the detection limit within 15 min when treated with 30 ppmv of ClO_2 gas at 70% RH. Exposure to 30 ppmv of ClO_2 gas under conditions of 90% RH reduced *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* to below the detection limit within 10 min treatment.

Table II-6. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes after 1 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.54(0.32)Aa[b]	0.51(0.43)Aa	0.64(0.33)Aa	0.79(0.35)Aa	0.85(0.25)Aa
	70	0.53(0.50)Aa	0.78(0.25)Aa	1.05(0.48)Aa	1.22(0.44)Aab	1.22(0.38)Aab
	90	0.63(0.38)Aa	0.97(0.19)ABa	1.31(0.17)BCa	1.55(0.15)Cb	1.64(0.19)Cb
S. Typhimurium	50	0.58(0.30)Aa	0.62(0.41)Aa	0.70(0.38)Aa	0.88(0.17)Aa	0.98(0.28)Aa
	70	0.63(0.14)Aa	0.65(0.29)Aa	0.77(0.56)Aa	1.02(0.42)Aa	1.11(0.50)Aa
	90	0.45(0.51)Aa	0.68(0.40)Aa	0.87(0.33)Aa	1.03(0.19)Aa	1.24(0.56)Aa
L. monocytogenes	50	0.64(0.31)Aa	0.63(0.42)Aa	0.78(0.44)Aa	0.87(0.39)Aa	0.87(0.40)Aa
	70	0.72(0.28)Aa	0.75(0.34)Aa	0.85(0.16)Aa	0.97(0.32)Aa	1.02(0.15)Aa
	90	0.43(0.19)Aa	0.54(0.48)Aa	0.72(0.28)Aa	0.86(0.44)Aa	0.96(0.16)Aa

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-7. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes after 5 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.53(0.13)Aa[b]	0.57(0.10)Aa	0.58(0.07)Aa	0.85(0.10)Ba	1.17(0.16)Ca
	70	0.78(0.14)Aa	0.99(0.26)Aab	1.06(0.11)Ab	1.55(0.35)Ba	1.81(0.08)Bb
	90	0.69(0.48)Aa	1.15(0.29)ABb	1.28(0.27)ABb	1.74(0.67)BCa	2.15(0.38)Cb
S. Typhimurium	50	0.62(0.20)Aa	0.79(0.02)ABa	0.82(0.04)ABa	0.91(0.21)ABa	0.97(0.17)Ba
	70	0.53(0.17)Aa	0.77(0.21)ABA	0.85(0.08)Ba	1.26(0.20)Ca	1.54(0.11)Cb
	90	0.50(0.09)Aa	0.72(0.08)Ba	1.22(0.03)Cb	1.81(0.09)Db	2.14(0.16)Ec
L. monocytogenes	50	0.68(0.07)Aa	0.81(0.05)Aa	0.93(0.40)Aa	0.99(0.07)Aa	1.03(0.34)Aa
	70	0.86(0.16)Aa	1.20(0.10)ABc	1.34(0.27)BCa	1.51(0.30)BCb	1.62(0.19)Cb
	90	0.64(0.16)Aa	1.03(0.03)Bb	1.45(0.09)Ca	1.93(0.18)Dc	2.37(0.07)Ec

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-8. Log reductions[a] of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on tomatoes after 10 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.68(0.23)Aa[b]	0.80(0.28)Aa	0.87(0.25)Aa	1.07(0.26)Aa	1.11(0.24)Aa
	70	0.86(0.15)Aa	0.97(0.21)ABab	1.29(0.19)Ba	1.64(0.18)Cb	1.97(0.20)Cb
	90	0.97(0.05)Aa	1.56(0.42)Ab	2.34(0.32)Bb	2.98(0.25)Bc	4.26(0.65)Cc
S. Typhimurium	50	0.62(0.02)Aa	0.82(0.35)ABa	1.11(0.38)ABa	1.22(0.36)ABa	1.33(0.42)Ba
	70	0.62(0.18)Aa	0.95(0.05)ABa	1.19(0.31)BCa	1.43(0.24)Ca	1.92(0.17)Da
	90	0.89(0.34)Aa	1.36(0.32)ABa	1.92(0.36)Bb	2.61(0.24)Cb	4.33(0.40)Db
L. monocytogenes	50	0.69(0.38)Aa	0.74(0.03)Aa	0.98(0.48)Aa	1.07(0.36)Aa	1.10(0.52)Aa
	70	0.70(0.29)Aa	0.78(0.24)Aa	0.94(0.24)ABa	1.27(0.24)BCa	1.46(0.25)Ca
	90	0.61(0.28)Aa	0.82(0.24)Aa	1.44(0.27)Ba	2.47(0.30)Cb	3.30(0.47)Db

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-9. Log reductions[a] of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on tomatoes after 20 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.59(0.09)Aa[b]	0.57(0.13)Aa	0.79(0.18)ABa	0.84(0.09)BCa	1.07(0.14)Ca
	70	0.43(0.12)Aa	1.09(0.28)Bb	1.99(0.43)Cb	3.13(0.55)Db	4.39(0.08)Eb
	90	0.45(0.15)Aa	1.41(0.21)Bb	3.60(0.53)Cc	> 6.74Dc	> 6.74Dc
S. Typhimurium	50	0.64(0.17)Aa	0.70(0.05)Aa	0.88(0.06)ABa	1.10(0.21)BCa	1.35(0.29)Ca
	70	0.47(0.23)Aa	1.06(0.30)Bab	1.68(0.13)Cb	2.51(0.23)Db	3.88(0.30)Eb
	90	0.55(0.09)Aa	1.38(0.31)Bb	3.50(0.49)Cc	> 6.93Dc	> 6.93Dc
L. monocytogenes	50	0.66(0.19)Aa	0.94(0.13)ABa	0.96(0.09)ABa	1.07(0.43)ABa	1.23(0.09)Ba
	70	0.43(0.43)Aa	0.87(0.51)ABa	1.61(0.57)BCa	2.17(0.19)Cb	3.06(0.55)Db
	90	0.49(0.52)Aa	1.51(0.30)Ba	3.15(0.45)Cb	> 5.87Dc	> 5.87Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table II-10. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes after 30 ppmv ClO_2 gas treatments.

Bacteria	RH (%)	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	50	0.70(0.09)Aa[b]	0.75(0.03)Aa	1.14(0.12)Ba	1.19(0.24)Ba	1.39(0.24)Ba
	70	0.36(0.26)Aa	0.85(0.34)Aa	2.83(0.56)Bb	> 6.85Cb	> 6.85Cb
	90	0.89(0.03)Aa	2.02(0.18)Bb	> 6.85Cc	> 6.85Cb	> 6.85Cb
S. Typhimurium	50	0.75(0.11)Aa	0.92(0.17)ABa	1.13(0.10)ABa	1.08(0.15)ABa	1.22(0.38)Ba
	70	0.73(0.44)Aa	1.17(0.37)Aa	2.48(0.50)Bb	> 7.01Cb	> 7.01Cb
	90	0.89(0.03)Aa	2.02(0.18)Bb	> 6.90Cc	> 6.90Cb	> 6.90Cb
L. monocytogenes	50	0.44(0.10)Aab	0.71(0.22)ABa	0.89(0.26)Ba	1.07(0.23)Ba	1.52(0.29)Ca
	70	0.24(0.02)Aa	0.70(0.15)Aa	2.28(0.59)Bb	> 5.49Cb	> 5.49Cb
	90	0.68(0.29)Ab	1.95(0.19)Bb	> 5.78Cc	> 5.78Cb	> 5.78Cb

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after ClO_2 gas treatment. Values are means (n = 3) and values in parentheses represent the standard deviations (SD) of the means.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

ClO_2 concentration on sample surfaces after treatment. Fig. II-2 shows ClO_2 concentration on spinach surfaces treated with 10 and 30 ppmv of ClO_2 gas under different RH conditions (50, 70, and 90%) for 20 and 15 min, respectively. No significant differences ($p > 0.05$) in ClO_2 concentration were observed between samples treated with 10 and 30 ppmv ClO_2 gas under conditions of 50 and 70% RH. At a given treatment concentration, ClO_2 concentration on spinach surfaces significantly ($p < 0.05$) increased with increasing RH to 90%.

Fig. II-3 shows ClO_2 concentration on tomato surfaces treated with 10, 20, and 30 ppmv of ClO_2 gas under conditions of 50, 70, and 90% RH for 20, 15, and 10 min, respectively. At a given treatment concentration, ClO_2 concentration on tomato surfaces significantly ($p < 0.05$) increased with increasing RH, except for those treated with 10 ppmv of ClO_2 gas at 50 and 70% RH. No significant differences ($p > 0.05$) in ClO_2 concentration were observed between samples treated with ClO_2 gas under conditions of 50% RH and the control.

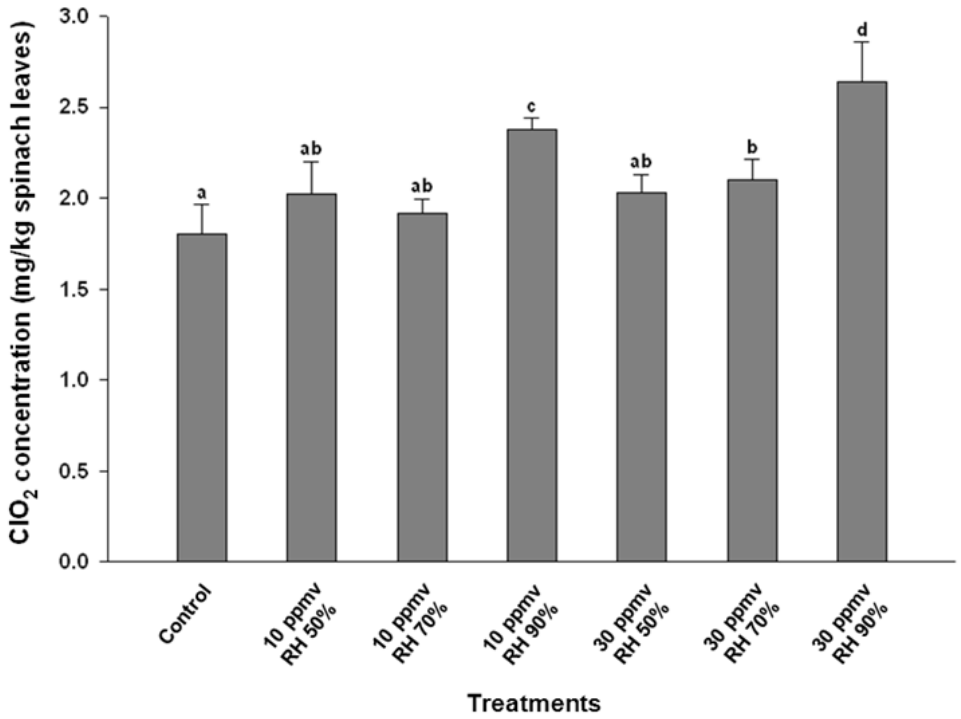


Fig. II-2. ClO_2 concentration on spinach surfaces immediately following treatment with 10 or 30 ppmv ClO_2 gas under 50, 70, or 90% RH for 20 or 15 min, respectively. Values with different lowercase letters were significantly different ($p < 0.05$). Values are expressed as the mean values of triplicate experiments, with error bars.

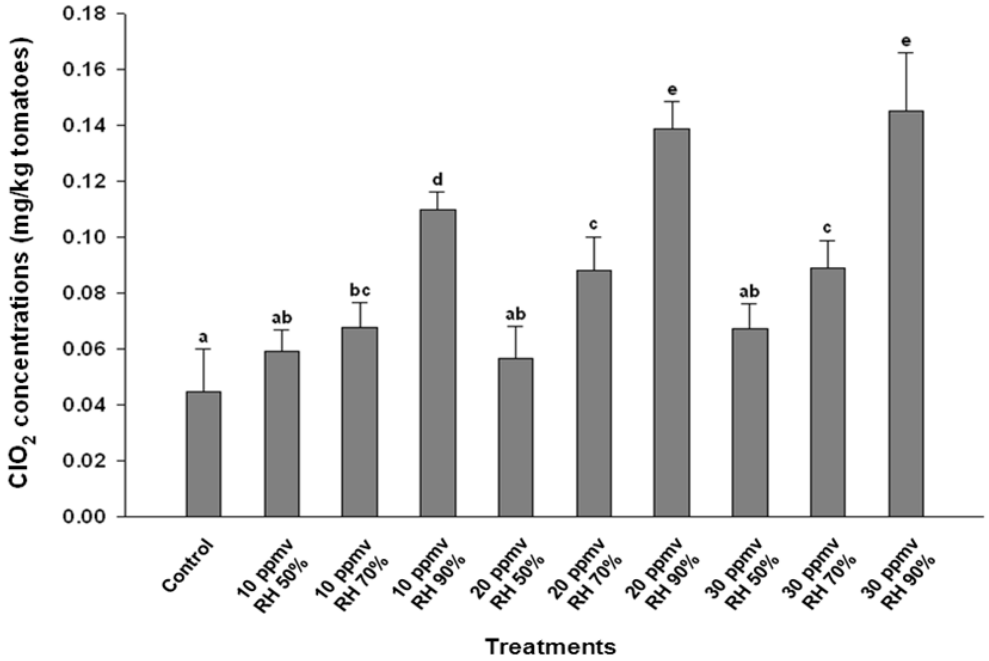


Fig. II-3. ClO_2 concentration on tomato surfaces immediately following treatment with 10, 20, or 30 ppmv ClO_2 gas under 50, 70, or 90% RH for 20, 15, or 10 min, respectively. Values with different lowercase letters were significantly different ($p < 0.05$). Values are expressed as the mean values of triplicate experiments, with error bars.

Effects of ClO_2 gas treatment on the quality of spinach leaves. No significant ($p > 0.05$) differences in Hunter's color values (L^*, a^*, b^*) were observed between untreated spinach leaves (control) and those treated with 1 to 30 ppmv of ClO_2 gas during storage at 4 ℃ for 7 days (data not shown). However, differences in color values between control and spinach leaves exposed to 50 ppmv of ClO_2 gas were observed during storage (Table II-11). The L^* value of spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) increased after 7 days. The a^* value of spinach leaves treated with 50 ppmv of ClO_2 gas under RH 70 and 90% conditions significantly ($p < 0.05$) decreased after 3 days. After 7 days of storage, the a^* value of all spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) decreased. The b^* value of spinach leaves treated with 50 ppmv of ClO_2 gas under 70 and 90% RH significantly ($p < 0.05$) increased after 3 days, and the b^* value of all spinach leaves treated with 50 ppmv of ClO_2 gas significantly ($p < 0.05$) increased after 7 days. There were no significant ($p > 0.05$) differences in texture between untreated spinach leaves (control) and those treated with ClO_2 gas during storage at 4 ℃ for 7 days (Table II-12).

Table II-11. Color changes[a] of spinach leaves during 4 °C storage for 7 days after treatment with 50 ppmv ClO_2 gas for 20 min.

Day	Treatment			
	Control	RH 50%	RH 70%	RH 90%
			*L**	
0	33.66±0.91A[b]	33.02±0.75A	33.45±1.37A	33.05±1.00A
3	33.54±0.87A	33.66±1.06A	34.32±0.36A	33.60±0.79A
7	33.11±0.43A	36.58±0.85B	36.33±0.51B	36.31±0.69B
			*a**	
0	-7.32±0.97A	-7.31±0.51A	-7.27±0.33A	-7.20±0.69A
3	-7.36±0.61A	-7.32±0.35A	-8.65±0.33B	-8.75±0.58B
7	-7.17±0.31A	-8.97±0.71C	-8.78±0.64BC	-8.39±0.42BC
			*b**	
0	11.70±0.62A	11.03±0.92A	11.28±0.80A	11.36±0.74A
3	11.22±0.67A	11.42±0.62AB	13.30±0.40BC	13.00±0.65C
7	10.49±1.38A	14.34±0.90B	14.36±0.55B	14.29±0.50B

[a] Color parameters are lightness (*L**), redness (*a**), and yellowness (*b**).

[b] Means ± standard deviations from three replications. Within the same storage time, means with different uppercase letters within a row are significantly different ($p < 0.05$).

Table II-12. Maximum force (N) required for breakage of spinach leaves during 4 °C storage for 7 days after treatment with ClO_2 gas (50 ppmv) for 20 min under differing levels of RH.

Days	Maximum force (N)			
	Control	RH 50%	RH 70%	RH 90%
0	51.41±2.20A[a]	50.35±1.60A	53.27±1.99A	52.46±1.32A
3	51.53±1.32A	50.85±0.01A	49.17±1.15A	52.52±0.10A
7	51.08±0.02A	50.37±0.39A	50.83±1.79A	51.80±2.42A

[a] Means ± standard deviations from three replications. Within the same storage time and type of vegetable, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

Effects of ClO_2 gas treatment on the quality of tomatoes. The *L** value of tomatoes decreased while the *a** and *b** values increased during storage (Table II-13). However, no significant ($p > 0.05$) differences in Hunter's color values (*L**, *a**, *b**) between untreated samples (control) and those treated with ClO_2 gas (30 ppmv) for 20 min were observed during 7 days storage at 12 ℃. The effects of exposure to ClO_2 gas (30 ppmv) for 20 min on the texture of tomatoes during storage are shown in Table II-14. There were no significant ($p > 0.05$) differences in texture between control and treated tomatoes during storage at 12 ℃ for 7 days.

Table II-13. Color changes[a] of tomatoes during 12 °C storage for 7 days after treatment with 30 ppmv ClO_2 gas for 20 min.

Days	Treatment			
	Control	RH 50%	RH 70%	RH 90%
			*L**	
0	42.12±0.20A[b]	40.93±0.28A	42.07±0.74A	42.00±0.44A
2	40.35±1.79A	39.19±0.19A	39.49±1.26A	40.49±0.78A
4	37.96±1.17A	37.71±1.01A	37.94±0.40A	38.33±1.15A
7	38.19±0.76A	38.50±0.93A	39.06±0.51A	38.47±0.25A
			*a**	
0	19.54±1.18A	19.06±1.56A	18.87±0.83A	18.63±0.85A
2	20.41±1.80A	19.82±0.79A	19.51±0.74A	19.20±1.04A
4	22.19±1.42A	20.61±1.33A	20.27±0.33A	20.32±0.65A
7	21.38±1.07A	21.04±0.86A	19.23±0.63A	20.95±0.50A
			*b**	
0	24.01±0.86A	23.11±0.83A	23.03±0.22A	23.75±0.31A
2	25.99±0.60A	26.26±0.61A	24.50±0.83A	25.20±0.65A
4	28.39±0.87A	26.24±0.44A	27.17±0.40A	28.82±1.81A
7	28.24±0.87A	26.81±0.93A	27.40±1.30A	26.84±1.72A

[a] Color parameters are lightness (*L**), redness (*a**), and yellowness (*b**).

[b] Within the same storage interval, means with different uppercase letters within a row are significantly different ($p < 0.05$).

Table II-14. Maximum force (N) required for breakage of tomatoes during 12 °C storage for 7 days after treatment with ClO_2 gas (30 ppmv) for 20 min under differing levels of RH.

Days	Maximum force (N)			
	Control	RH 50%	RH 70%	RH 90%
0	12.87±0.52A[a]	12.94±0.59A	13.16±0.13A	13.53±1.21A
2	12.40±0.25A	12.56±0.36A	12.44±0.57A	12.91±0.38A
4	11.87±0.85A	11.81±0.82A	11.92±0.88A	12.59±0.33A
7	12.06±0.32A	12.29±0.01A	12.19±0.56A	12.14±0.39A

[a] Means ± standard deviations from three replications. Within the same storage interval, means followed by the same uppercase letters within a row were not significantly different ($p > 0.05$).

II-1.4. Discussion

In the present study, ClO_2 gas showed a significant antimicrobial effect against foodborne pathogens on spinach leaves and tomatoes. Several sanitation methods have been evaluated to inactivate foodborne pathogens on spinach leaves. Treatment with sodium hypochlorite (100 ppm) for 5 min reduced levels of *E. coli* O157:H7 by 1.1 log CFU/g (Lee and Baek, 2008). Guentzel et al. (2008) reported a dipping treatment of spinach at 100 and 120 ppm total residual chlorine for 10 min resulted in a 4.0–5.0 log CFU/ml reduction of bacterial counts for *E. coli*, *S.* Typhimurium, *Staphylococcus aureus*, *L. monocytogenes*, and *Enterococcus faecalis*. Lactic, citric, malic, tartaric, and acetic acid (2%) resulted in 1.5 to 1.8 log CFU/g reduction of *E. coli* O157:H7 on baby spinach after 5 min of treatment (Huang and Chen, 2011). Neal et al. (2012) evaluated the antimicrobial effect of ClO_2 gas in inactivating *Salmonella* and *E. coli* O157:H7 on spinach leaves using a ClO_2 gas generating sachet. *Salmonella* and *E. coli* O157:H7 on spinach leaves exposed to 2.1 mg/L ClO_2 gas for 1 h were reduced by 0.6 and 0.7 log CFU/g, respectively.

In case of tomatoes, several studies have reported antimicrobial effects of ClO_2 gas on produce including tomatoes. Treatment with 10 mg/l ClO_2 gas for 120 s reduced levels of *Salmonella* on Roma tomatoes by 3.86 log CFU/cm^2 (Trinetta et al., 2010). After exposure to 0.5 mg/l ClO_2 gas for 12 min, more than a 5 log reduction in *Salmonella* and *L. monocytogenes* was observed on tomato skin

surfaces (Bhagat et al., 2010). Sy et al. (2005) reported that 4.1 mg/l ClO_2 gas resulted in a 4.33 log reduction of *Salmonella* spp.

Han et al. (2001a) reported that high RH enhanced the efficacy of ClO_2 gas in inactivating *E. coli* O157:H7 on green peppers. When green peppers were treated with 0.3 mg/L ClO_2 gas at 15 ℃, reductions increased from 1.93 to 4.00 log CFU/5g as RH increased from 55 to 95%. Growth of *Lactobacillus buchneri* on stainless steel strips after treatment with 8 mg/L ClO_2 gas for 10 min decreased as RH increased from 56 to 94% (Han et al., 1999). Regarding this synergistic relation between ClO_2 gas and RH, most studies have evaluated the antimicrobial effect of ClO_2 gas conducted under conditions of high RH (> 80%) (Bhagat et al., 2011; Gómez-López et al., 2008; Guentzel et al., 2008; Popa et al., 2007; Vandekinderen et al, 2009).

However, little information is available on the inactivation tendency of ClO_2 gas according to levels of RH and treatment time. This may be an important factor for practical application of ClO_2 gas by the food processing industry. The results of this study showed that treatment with ClO_2 gas under 90% RH caused more significant ($p < 0.05$) reductions in levels of three foodborne pathogens than ClO_2 gas treatment under 50 and 70% RH at the same ClO_2 gas concentrations. Also, differences in reduction levels between treatments under 90% RH and those under 50 and 70% RH increased as ClO_2 gas concentration increased. Generally, no significant differences ($p > 0.05$) in reduction levels of the three foodborne pathogens on spinach leaves

were observed between 50 and 70% RH. However, significant differences ($p < 0.05$) in inactivation levels of the three foodborne pathogens on tomatoes were observed between 50 and 70% RH as ClO_2 gas concentration and treatment time increased.

Differences in inactivation levels may due to the different solubility of ClO_2 gas under different levels of RH. ClO_2 gas acts similar to aqueous ClO_2 for inactivating microorganisms due to its high solubility in water (Linton et al., 2006). To determine the quantity of solubilized ClO_2 gas on the surface of spinach leaves and tomatoes, the DPD method used. There were no significant differences ($p > 0.05$) in ClO_2 concentration on spinach surfaces which treated with 10 and 30 ppmv ClO_2 gas under conditions of 50 and 70% RH. At a given treatment concentration, ClO_2 concentration on spinach leaves significantly ($p < 0.05$) increased with increasing RH to 90%. This tendency follows the inactivation patterns of foodborne pathogens under different RH conditions. For example, inactivation of the three pathogens by 10 ppmv ClO_2 gas treatment for 20 min was not significantly different ($p > 0.05$) from that of 30 ppmv of ClO_2 for 15 min under conditions of 50 and 70% RH. As shown in Fig. II-2, no significant differences were observed ($p > 0.05$) between ClO_2 concentration on spinach surfaces treated with 10 ppmv of ClO_2 gas under 50 and 70% RH for 20 min and those treated with 30 ppmv of ClO_2 gas at 50 and 70% RH for 15 min. These results indicate that there are close correlations between log reductions of pathogens and ClO_2 concentration on spinach surfaces.

Similar results were observed on tomatoes. For example, under conditions of 70 and 90% RH, inactivation of the three pathogens by 20 ppmv ClO_2 gas treatment for 15 min was not significantly different ($p > 0.05$) from that of 30 ppmv of ClO_2 for 10 min. As shown in Fig. II-3, no significant differences were observed ($p > 0.05$) between ClO_2 concentration on tomato surfaces treated with 20 ppmv of ClO_2 gas under 70 and 90% RH for 15 min and those treated with 30 ppmv of ClO_2 gas at 70 and 90% RH for 10 min.

The color of spinach leaves treated with 50 ppmv of ClO_2 gas gradually changed during storage. Also, color changes occurred rapidly in spinach leaves treated under conditions of high RH. Changing color of spinach leaves during storage may be due to the high oxidation capacity of ClO_2 gas (Mahmoud et al., 2008). Discoloration of fresh produce following ClO_2 gas treatment has been reported in several studies (Guentzel et al., 2008; Mahmoud and Linton, 2008; Sy et al., 2005). Therefore, treatment conditions should be optimized considering the inactivation effect and desired quality of the food product.

In conclusion, ClO_2 gas showed significant antimicrobial effect against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves and tomatoes, and significant differences ($p < 0.05$) were observed between inactivation levels under different RH conditions as ClO_2 gas concentration and treatment time increased. Variations in RH have great effect on the solubilization of ClO_2 gas on spinach and tomato surfaces considering that ClO_2 concentration on those produce

surfaces increased with increasing RH. Also, the amount of ClO_2 concentration on spinach and tomato surfaces is correlated with the level of inactivation of pathogens. The results of this study provide insights for predicting inactivation patterns of foodborne pathogens by ClO_2 gas for practical application by the fresh produce industry.

II-2. Effect of surface characteristics of produce and food contact surfaces on the inactivation of foodborne pathogens by chlorine dioxide gas

II-2.1. Introduction

In recent years chlorine dioxide (ClO_2) gas has emerged as a promising non-thermal sanitizing technology for fresh produce (Bhagat et al., 2010). ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum, and its efficacy is not greatly affected by pH or organic matter. Also, ClO_2 does not react with nitrogen compounds to form chloramines (Aieta et al., 1984). The mechanism of inactivation by ClO_2 has been postulated by several studies. Damage to protein synthesis and increased permeability of the outer cell membrane is the most widely accepted antimicrobial mechanism of ClO_2 (Aieta and Berg, 1986; Roller et al., 1980).

ClO_2 gas has been evaluated to inactivate foodborne pathogens on fresh produce such as spinach (Neal et al., 2012; Park and Kang, 2015), potatoes (Wu and Rioux, 2010), apples (Du et al., 2002), tomatoes (Bhagat et al., 2010; Trinetta et al., 2013), lettuce (Mahmoud and Linton, 2008), mung bean sprouts (Prodduk et al., 2014), cabbage (Sy et al., 2005), cantaloupe (Mahmoud et al., 2008), and strawberries (Han et al., 2004). Also, the antimicrobial effect of ClO_2 gas against foodborne pathogens on food contact surfaces such as wood, plastic (Han et al., 2003), stainless steel (Trinetta et al., 2012; Vaid et al., 2010), polyvinyl chloride, and glass (Li et al., 2012; Morino et al., 2011) have been evaluated.

Surface properties could influence bacterial inactivation from a surface (Wang et al., 2009). Surface hydrophobicity, surface constitutional characteristics, and surface

topography could describe surface properties (Wang et al., 2009). Surface hydrophobicity relates to surface structures and surface chemical composition, and influences surface hydration (Vacheethasanee et al., 1998). Also, surface hydrophobicity could influence the distribution and attachment of bacteria on surfaces (Harkes et al., 1991; Syamaladevi et al., 2013). Surface topography has been known to influence bacterial attachment to and removal from a surface (Faille et al., 2000; Jullien et al., 2002). The influence of surface hydrophobicity and surface roughness on microbial inactivation has been reported by some studies. Wang et al. (2009) reported that a positive linear relationship between surface roughness values and residual bacterial populations was observed after washing treatments. Increasing the surface roughness appeared to induce lower inactivation of *Listeria innocua* on food packaging materials (Ringus and Moraru, 2013). Conversely, Fernandes et al. (2014) observed that roughness and hydrophobicity of the fruit surface did not affect the efficiency of sanitation treatments on removal of *Salmonella* Typhimurium. Jullien et al. (2002) indicated that it was difficult to link the hygienic status of stainless steel to surface roughness values.

Gas concentration, treatment time, relative humidity (RH), and temperature could affect the antimicrobial effect of ClO_2 gas, and especially, the combination of gas concentration and RH shows a synergistic effect (Han et al., 2001a). However, there are no studies considering the influence of surface properties of produce and food contact surfaces on the inactivation efficacy of ClO_2 gas. Also, comparative

data for different produce and food contact surfaces subjected to the same treatment are not readily available.

The objective of this study was to examine how surface properties (hydrophobicity and roughness) of produce and food contact surfaces influence the antimicrobial effect of ClO_2 gas against *Escherichia coli* O157:H7, *S.* Typhimurium, and *Listeria monocytogenes* on these selected surfaces.

II-2.2. Materials and Methods

Bacterial strains. Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea), for this study.

Sample preparation. Carrots, kale, cabbage, spinach, apples, tomatoes, and paprika were purchased from a local market (Seoul, South Korea). These produce were washed in running water and dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h before experiments to remove surface moisture. Produce surfaces were cut into 5 × 2 cm pieces. Food contact surfaces tested included Teflon (Gongguone, Goyang-si, South Korea), silicon (Jun Sangsa, Seoul, South Korea), rubber (Chehyung, Seoul, South Korea), polyvinyl chloride (PVC) (Kahee, Incheon, South Korea), type 304 stainless steel (SS) with 2B or No.4 finish (Ian industry, Ansan-si, South Korea), and glass (Corning Inc., NY, USA). These materials were cut into coupons (5 × 2 cm), immersed in 70% ethanol for 20 min, and rinsed with distilled water. After washing, coupons were dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h to remove surface moisture. Each coupon was used only once in order to avoid any surface changes.

Surface hydrophobicity measurement. The hydrophobicity of produce and food contact surfaces was evaluated by water contact angle measurements. Water contact angle was measured by the sessile drop method using a contact angle goniometer (DSA 100, KRUSS, Germany) equipped with a camera. Small drops (3 μl) of distilled water were deposited onto the produce and food contact surfaces described above using a microliter syringe and a 0.5-mm diameter needle at room temperature (22 ± 2 ℃). Contact angle measurements were conducted for less than 30 s to avoid changes in the tested surface. Ten data points were taken for each sample (n = 10).

Surface roughness measurement. White light scanning interferometry (WLSI) was used to acquire topographic images and surface roughness values of produce and food contact surfaces. Glass surface was coated with platinum by ion sputtering to get a reflective surface. Samples were directly mounted on the stage of a noncontact three-dimensional surface profiler (NanoView-E1000, NanoSystem, Daejeon, South Korea). Topographic images of 125 × 95 μm areas were acquired from each sample. Height profiles were expressed in the topographic images (3D) by the color scale. The R_a (arithmetic mean roughness) and R_q (root mean squared roughness) values were calculated from 10 scan area (125 × 95 μm) of each sample using a software package (NanoMap Version 2.5.17.0; NanoSystem).

Culture preparation and sample inoculation. All strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were cultured individually in 10 ml of tryptic

soy broth (TSB; Difco, Sparks, MD, USA) at 37 ℃ for 24 h, harvested by centrifugation at 4000 × g for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile distilled water, corresponding to ca. 10^7-10^8 CFU/ml. Mixed culture cocktails were prepared by blending together equal volumes of all test strains.

Prepared produce and food contact surface coupons were placed on aluminum foil in a laminar flow hood, and 0.1 ml of culture cocktail was inoculated onto each sample by depositing droplets with a micropipettor at 14-16 locations. After inoculation, samples were dried in a laminar flow biosafety hood for 1 h at 22 ± 2 ℃.

ClO_2 gas treatment. ClO_2 gas treatment was conducted in a treatment system described previously (Park and Kang, 2015). Inoculated samples were placed in the treatment chamber and covered with a plastic lid. Samples were treated with 20 ppmv ClO_2 gas for 5, 10, and 15 min at 22 ± 2 ℃. The RH of the treatment chamber was adjusted to 90% with an accuracy of ± 2%. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and samples were exposed to the treatment. These experiments were repeated three times.

Bacterial enumeration. Treated produce samples were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 30 ml of neutralizing buffer (Difco). Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. Treated food contact surface coupons were transferred to sterile 50-ml conical centrifuge tubes containing

30 ml of neutralizing buffer and 3 g of glass beads (425-600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then vortexed for 2 min. After homogenization, 1 mL aliquots of the sample were tenfold serially diluted in 9 mL of BPW, and 0.1 ml aliquots of sample or diluents were spread-plated onto selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Where low numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates to lower the detection limit. The plates were incubated at 37 ℃ for 24–48 h. After incubation, colonies were counted and calculated as log CFU/cm^2.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$. Correlation coefficients between surface properties and the log reduction of pathogens were calculated using SPSS statistical software (version 22; SPSS, Chicago, IL).

II-2.3. Results

Contact angle of produce and food contact surfaces. Table II-15 shows water contact angles of produce and food contact surfaces. For all selected produce, water contact angles were greater than 65°, indicating their hydrophobic nature. Kale (122.7°) and carrots (119.3°) showed the highest water contact angles, followed by cabbage (111.0°), spinach (101.8°), apples (99.1°), tomatoes (92.4°), and paprika (87.2°). Among food contact materials, silicon (113.4°) represented the highest water contact angle, followed by Teflon (109.1°), rubber (105.7°), stainless steel 2B finish (SS 2B) (97.8°), PVC (93.0°), and stainless steel No.4 finish (SS No.4) (84.4°). Water contact angle of glass was 49.8°, indicating its hydrophilic nature.

Table II-15. Contact angle of the different produce and food contact surfaces.

Produce surfaces	Contact angle (°)	Food contact surfaces	Contact angle (°)
Kale	122.7±4.3A[a]	Silicon	113.4±1.4A
Carrots	119.3±2.8A	Teflon	109.1±1.5B
Cabbage	111.0±3.9B	Rubber	105.7±1.1B
Spinach	101.8±2.8C	Stainless steel 2B	97.8±1.5C
Apples	99.1±4.3C	PVC	93.0±2.7D
Tomatoes	92.4±1.6D	Stainless steel No.4	84.4±1.2E
Paprika	87.2±1.8D	Glass	49.8±3.6F

[a] Means with different uppercase letters within a column are significantly different ($p < 0.05$).

Roughness of produce and food contact surfaces. The surface roughness parameters of produce and food contact surfaces are represented in Table II-16. R_a and R_q values of carrots (2.08 and 2.56 µm), spinach (1.77 and 2.19 µm), and kale (1.54 and 1.84 µm) were greater than those of other produce. Cabbage represented moderate R_a and R_q values (0.99 and 1.22 µm) among the 7 types of produce. Apples, tomatoes, and paprika had the smoothest surfaces, which showed 0.45, 0.31, and 0.60 for R_a and 0.57, 0.38, and 0.74 for R_q, respectively. Among food contact surfaces, silicon showed the highest R_a (0.57 µm) and R_q (0.70 µm) values, followed by rubber (0.28 and 0.36 µm), Teflon (0.21 and 0.27 µm), SS 2B (0.16 and 0.21 µm), SS No.4 (0.14 and 0.18 µm), PVC (0.03 and 0.04 µm), and glass (0.003 and 0.004 µm).

Table II-16. Surface roughness parameters of produce and food contact surfaces.

Produce surfaces	Surface roughness parameters (μm)[a]		Food contact surfaces	Surface roughness parameters (μm)	
	R_a	R_q		R_a	R_q
Kale	1.54±0.10B[b]	1.84±0.13B	Silicon	0.57±0.10A	0.70±0.12A
Carrots	2.08±0.47A	2.56±0.49A	Teflon	0.21±0.08BC	0.27±0.09BC
Cabbage	0.99±0.25C	1.22±0.33C	Rubber	0.28±0.09B	0.36±0.12B
Spinach	1.77±0.22AB	2.19±0.30AB	Stainless steel 2B	0.16±0.02C	0.21±0.03C
Apples	0.45±0.19D	0.57±0.23D	PVC	0.03±0.01D	0.04±0.01D
Tomatoes	0.31±0.05D	0.38±0.05D	Stainless steel No.4	0.14±0.03CD	0.18±0.03CD
Paprika	0.60±0.16CD	0.74±0.19CD	Glass	0.003±0.0007E	0.004±0.0009E

[a] R_a, arithmetic mean roughness; R_q, root mean squared roughness.
[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$).

ClO_2 gas inactivation of foodborne pathogens. Tables II-17 to 19 show log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on produce surfaces after treatment with 20 ppmv ClO_2 gas. As treatment time increased, differences in degree of inactivation of the three pathogens were observed. Low inactivation levels of *E. coli* O157:H7 were achieved on kale and carrots, with 1.38 and 1.50 log reductions after 15 min treatment, respectively. Levels of *E. coli* O157:H7 cells on cabbage, spinach, apples, and tomatoes were reduced by 15 min treatment with ClO_2 gas, showing 2.62, 3.41, 3.95, and 4.72 log reductions, respectively. The highest inactivation of *E. coli* O157:H7 was observed on paprika with 5.43 log reduction after 15 min treatment. *S.* Typhimurium and *L. monocytogenes* showed reduction patterns similar to *E. coli* O157:H7. Treatment with ClO_2 gas for 15 min caused 1.33, 1.02, 2.31, 3.41, 3.95, 4,69, and 5.29 log reductions of *S.* Typhimurium, and 1.05, 0.86, 2.07, 2.97, 3.50, 4.12, and 4.91 log reductions of *L. monocytogenes* on kale, carrots, cabbage, spinach, apples, tomatoes, and paprika, respectively.

ClO_2 gas (20 ppmv) treatment for 15 min was less effective for inactivating *E. coli* O157:H7 on silicon and Teflon, with reductions of 1.44 and 1.26 log CFU/cm^2, respectively (Table II-20). The degree of log reduction of *E. coli* O157:H7 increased in the following order: rubber (2.24 log), SS 2B (2.75 log), PVC (3.00 log), and SS No.4 (3.43 log). The Highest inactivation level was achieved on glass with more than 6.81 log reduction. Similar inactivation patterns were observed for *S.*

Typhimurium and *L. monocytogenes*. Exposure to 20 ppmv ClO_2 gas for 15 min resulted in 0.96, 1.25, 1.90, 2.28, 2.44, 2.76, and more than 6.76 log reductions of *S.* Typhimurium, and 1.14, 1.25, 1.38, 1.81, 2.35, 2.64, and more than 5.91 log reductions of *L. monocytogenes* on silicon, Teflon, rubber, SS 2B, PVC, SS No.4, and glass, respectively (Table II-21, 22).

Table II-17. Log reduction[a] of *E. coli* O157:H7 on produce surfaces treated with 20 ppmv ClO_2 gas.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Kale	0.59±0.24Aa[b]	0.79±0.27Aa	1.38±0.33Ab
Carrots	0.65±0.31Aa	1.31±0.11ABab	1.50±0.57Ab
Cabbage	0.91±0.33Aa	1.75±0.17BCb	2.62±0.24Bc
Spinach	1.33±0.15Ba	2.13±0.55CDb	3.41±0.03Cc
Apples	1.39±0.07Ba	2.26±0.32CDb	3.95±0.41Cc
Tomatoes	1.33±0.14Ba	2.64±0.39Db	4.72±0.11Dc
Paprika	1.35±0.18Ba	3.42±0.44Eb	5.43±0.45Ec

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Table II-18. Log reduction[a] of *S.* Typhimurium on produce surfaces treated with 20 ppmv ClO_2 gas.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Kale	0.73±0.50ABa[b]	0.90±0.48Aa	1.33±0.52Aa
Carrots	0.49±0.34Aa	0.94±0.53Aa	1.02±0.11Aa
Cabbage	0.73±0.23ABa	1.12±0.35Aa	2.31±0.33Bb
Spinach	1.13±0.10ABCa	2.31±0.12Bb	3.41±0.18Cc
Apples	1.25±0.39BCa	2.02±0.21Bb	3.95±0.40Cc
Tomatoes	1.17±0.50ABCa	2.53±0.13Bb	4.69±0.29Dc
Paprika	1.59±0.37Ca	3.32±0.29Cb	5.29±0.44Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Table II-19. Log reduction[a] of *L. monocytogenes* on produce surfaces treated with 20 ppmv ClO_2 gas.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Kale	0.76±0.14Ba[b]	0.97±0.48ABa	1.05±0.55Aa
Carrots	0.22±0.22Aa	0.43±0.30Aab	0.86±0.34Ab
Cabbage	1.12±0.30BCDa	1.45±0.26BCa	2.07±0.16Bb
Spinach	1.02±0.12CDa	2.14±0.52CDb	2.97±0.44Cc
Apples	1.47±0.37CDEa	2.28±0.45Db	3.50±0.28CDc
Tomatoes	1.82±0.24Ea	2.57±0.02Db	4.12±0.19Dc
Paprika	1.53±0.30DEa	2.70±0.60Db	4.91±0.41Ec

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Table II-20. Log reduction[a] of *E. coli* O157:H7 on food contact surfaces after 20 ppmv ClO_2 gas treatments surfaces.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Silicon	0.50±0.54Aa[b]	0.57±0.54Aa	1.44±0.57Aa
Teflon	0.65±0.27Aa	0.87±0.39Aab	1.26±0.10Ab
Rubber	1.13±0.24ABa	1.46±0.04Ba	2.24±0.17Bb
Stainless steel 2B	1.11±0.35ABa	1.87±0.35BCb	2.75±0.39BCc
PVC	1.46±0.35Ba	2.09±0.14Cb	3.00±0.28CDc
Stainless steel No.4	1.51±0.47Ba	2.27±0.24Cb	3.43±0.37Dc
Glass	2.14±0.07Ca	5.62±0.44Db	> 6.81Ec

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Table II-21. Log reduction[a] of *S.* Typhimuruim on food contact surfaces after 20 ppmv ClO_2 gas treatments surfaces.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Silicon	0.42±0.32Aa[b]	0.58±0.39Aa	0.96±0.45Aa
Teflon	0.61±0.45ABa	0.84±0.19ABa	1.25±0.37ABa
Rubber	0.97±0.39ABa	1.23±0.23BCa	1.90±0.32BCb
Stainless steel 2B	0.92±0.37ABa	1.55±0.24Cab	2.28±0.49CDc
PVC	1.14±0.20BCa	1.53±0.20Ca	2.44±0.43CDc
Stainless steel No.4	1.07±0.50ABCa	1.67±0.35Ca	2.76±0.45Db
Glass	1.72±0.13Ca	5.44±0.28Db	> 6.76Ec

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Table II-22. Log reduction[a] of *L. monocytogenes* on food contact surfaces after 20 ppmv ClO_2 gas treatments surfaces.

Sample	Log reduction (CFU/cm^2)		
	Treatment time		
	5 min	10 min	15 min
Silicon	0.56±0.10Aa[b]	0.89±0.24Aab	1.14±0.24Ab
Teflon	0.93±0.45ABa	1.02±0.45Aa	1.25±0.42Aa
Rubber	0.90±0.26ABa	1.05±0.40Aa	1.38±0.31ABa
Stainless steel 2B	1.10±0.08BCa	1.23±0.21ABa	1.81±0.07Bb
PVC	1.45±0.10CDa	1.77±0.20Bab	2.35±0.48Cb
Stainless steel No.4	1.19±0.16BCa	1.78±0.38Bb	2.64±0.20Cc
Glass	1.68±0.09Da	> 5.91Cb	> 5.91Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a column are significantly different ($p < 0.05$). Means with different lowercase letters within a row are significantly different ($p < 0.05$).

Effect of surface properties on ClO_2 gas inactivation of foodborne pathogens. The correlation coefficients between surface properties and bacterial log reductions (after exposure to 20 ppmv ClO_2 gas for 15 min) are shown in Table II-23. Contact angles of produce surfaces were highly and negatively correlated with the log reductions of all three pathogens, showing a Pearson correlation coefficient (r) of -0.996, -0.991, and -0.992 for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Also, contact angles of food contact surfaces showed highly negative correlation with the bacterial log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, showing a Pearson correlation coefficient (r) of -0.990, -0.984, and -0.989 for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. The R_a and R_q values of produce surfaces were negatively correlated with the log reductions of the three pathogens, although the correlation coefficients were quite lower than those between contact angle and the bacterial log reduction. The R_a and R_q values of food contact surfaces were not significantly ($p > 0.05$) correlated with the log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*.

Table II-23. Correlation matrix of Pearson coefficients between surface properties and bacterial log reductions.

Bacterial reduction	Surface properties		
	Contact angle	R_a	R_q
Produce surfaces			
E. coli O157:H7	-0.996**[a]	-0.784*	-0.774*
S. Typhimurium	-0.991**	-0.790*	-0.782*
L. monocytogenes	-0.992**	-0.786*	-0.777*
Food contact surfaces			
E. coli O157:H7	-0.990**	NS	NS
S. Typhimurium	-0.984**	NS	NS
L. monocytogenes	-0.989**	NS	NS

[a] NS, not significant. *, level of significance at $p < 0.05$. **, level of significance at $p < 0.01$.

II-2.4. Discussion

Generally, surfaces with a water contact angle greater than 65° are considered to be hydrophobic (Vogler, 1998). For all the selected produce in this study, the water contact angles were greater than 65°, indicating their hydrophobic nature. For the food contact surfaces, all materials except glass represent hydrophobic nature. Contact angles of produce and food contact surfaces showed highly negative correlation with the log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*: that is, the more hydrophobic the surface, the less the reduction of the three pathogens. Differences in reduction levels according to hydrophobicity may due to different solubility of ClO_2 gas on the different surfaces. ClO_2 gas acts similarly to aqueous ClO_2 for inactivating bacteria due to its high water solubility (Linton et al., 2006). Therefore, it seems that as less ClO_2 gas was solubilized on the hydrophobic surfaces, lower reductions occurred on these surfaces.

Surface hydrophobicity may also influence the distribution and the attachment of bacteria on surfaces. It is likely that there was greater adherence of bacteria along with a more uniform and broader distribution of cells onto hydrophilic surfaces compared to hydrophobic surfaces. Boulange-Petermann et al. (Boulange-Petermann et al., 1993) found that microorganisms preferentially adhere to substrata of high wettability. Syamaladevi et al. (2013) reported that as pear surfaces were less hydrophobic than peach surfaces, a broader spatial distribution of bacterial cells

could be achieved. Therefore, the higher inactivation of *E. coli* cells on pear surfaces by UV-C could be attributed to the lower hydrophobicity of pear surfaces compared to peach. Also, the hydrophobic nature of raspberry surfaces was responsible for lower UV-C inactivation of *Penicillium expansum* populations in comparison to other fruits (apples, cherries, and strawberries) (Syamaladevi et al., 2015). A strong negative correlation between the water contact angle/hydrophobicity and the inactivation levels of the three pathogens in this study was consistent with these previous studies.

Another surface property that is expected to affect ClO_2 gas inactivation is surface roughness. For quantitative and qualitative surface analysis, white light scanning interferometry (WLSI) was used in this study. There are several surface roughness measuring instruments and methods, including surface profilometers and atomic force microscopy (AFM). However, it is difficult to measure the surface roughness of fruits and vegetables which are soft and deformable (Wang et al., 2009). The advantage of WLSI is that it executes noncontact scanning over the sample surface by white light to acquire topographic images, including a three-dimensional reconstruction of surface profiles (Kim et al., 2010b). It can be used to measure the profile of delicate membranes or soft materials without risk of damage (Gao et al., 2008). Also, surface roughness parameters, including R_a and R_q, are calculated from acquired topographic images of large scan areas, which are not usually available from AFM due to the limited scan areas.

Greater surface roughness could cause increased surface adhesion of bacteria due to increased surface area (Korber et al., 1997; Wang et al., 2009). Several studies have assumed that the lowest R_a values would be more hygienic (Bower et al., 1996; Faille et al., 2000; Fransisca and Feng, 2012; Percival, 1999). On the other hand, there are other investigations which have failed to demonstrate a clear relationship between R_a values and adhesion and/or removal of microorganisms (Barnes et al., 1999; Boulangé-Petermann et al., 1997). However, surface topography may play a major role in removal of bacteria, even if not on bacterial adhesion, by protecting bacterial cells from washing treatments (Scheuerman et al., 1998).

In the present study, the R_a and R_q values of produce surfaces were negatively correlated with the log reductions of three pathogens, although the correlation coefficients were quite lower than those between contact angle and bacterial log reduction. The R_a and R_q values of food contact surfaces were not significantly ($p > 0.05$) correlated with the log reductions of the three pathogens. Although the R_a and R_q values are useful for measuring gradual change in roughness due to cutting tool wear, these do not differentiate between crevices and valleys of a surface (Frank and Chmielewski, 2001). It seems that the existence of crevices and peaks of a surface was more important than the R_a and R_q values in the inactivation patterns of foodborne pathogens. Existence of many surface cavities larger than three pathogens'

cells could provide improved protection for foodborne pathogens against ClO_2 gas treatment.

In conclusion, this study showed that surface characteristics of produce and food contact surfaces have a great impact on the inactivation of foodborne pathogens by ClO_2 gas treatment. The results of this study indicate that surface hydrophobicity plays a more important role in bacterial inactivation from surfaces than surface roughness. Understanding surface characteristics is needed if this technology is to be commercialized. The results of this study are helpful for the food industry to establish ClO_2 gas treatment conditions.

II-3. Effect of temperature on solubility of ClO_2 gas and the inactivation of foodborne pathogens

II-3.1. Introduction

Chlorine dioxide (ClO_2) has emerged as a promising non-thermal sanitizing technology for produce in recent years (Bhagat et al., 2010). ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum (Trinetta et al., 2012). Its efficacy is not greatly affected by changes in pH or organic matter (Beuchat, 1998). Also, ClO_2 does not react with nitrogen compounds to form chloramines (Aieta et al., 1984). The most widely accepted antimicrobial mechanism of ClO_2 is damage to protein synthesis and increased permeability of the outer cell membrane (Aieta and Berg, 1986; Roller et al., 1980). Studies on application of ClO_2 gas to fresh produce, such as blueberries (Sun et al., 2014), spinach (Neal et al., 2012; Park and Kang, 2015), potatoes (Wu and Rioux, 2010), oranges (Bhagat et al., 2011), tomatoes (Bhagat et al., 2010; Trinetta et al., 2013), lettuce (Mahmoud and Linton, 2008), mung bean sprouts (Prodduk et al., 2014), carrots (Sy et al., 2005), and cantaloupe (Mahmoud et al., 2008) have been reported. Also, the antimicrobial effect of ClO_2 gas against pathogens on food contact surfaces such as stainless steel (Trinetta et al., 2012; Vaid et al., 2010), wood, plastic (Han et al., 2003), polyvinyl chloride, and glass (Li et al., 2012; Morino et al., 2012) have been evaluated.

The antimicrobial effect of ClO_2 gas is affected by several factors, primarily ClO_2 gas concentration and exposure time. Han et al (2001a) reported that the combination of relative humidity (RH) and gas concentration showed a synergistic

effect. Also, surface characteristics of treated samples including surface hydrophobicity and surface roughness could influence bacterial inactivation from a surface. Treatment temperature also could be an important factor affecting antimicrobial efficacy of ClO_2 gas since it could affect ClO_2 gas solubility. Cárdenas et al. (2011) reported that gaseous ozone treatment was more effective at 0 ℃ than 4 ℃ for reducing *E. coli* and total aerobic mesophilic heterotrophic microorganisms in beef. They concluded that the higher microbial reduction at lower temperature was due to higher solubility of ozone gas in the aqueous phase of the meat tissue. However, there have been no studies considering the influence of treatment temperature on the inactivation efficacy of ClO_2 gas. Although Han et al. (2001a) studied the effects and interactions of temperature (5 to 25 ℃) and RH (55 to 95%), absolute humidity (AH) should be used to compare the effect of different treatment temperatures on the solubility of ClO_2 gas.

The objective of this study was to determine how ClO_2 gas treatment temperature influences the antimicrobial effect of ClO_2 gas against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on produce and food contact surfaces. To assess the influence of treatment temperature on the solubility of ClO_2 gas, ClO_2 concentration on sample surfaces were analyzed.

II-3.2. Materials and Methods

Bacterial strains and culture preparation. Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104), and *L. monocytogenes* (ATCC 19111, ATCC 19115, and ATCC 15313) obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea) were used in this study. All strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were cultured individually in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 ℃ for 24 h and harvested by centrifugation at 4000 × g for 20 min at 4 ℃. The final pellets were resuspended in sterile buffered peptone water (BPW; Difco), corresponding to approximately 10^7–10^8 CFU/ml. Then, suspended pellets of the three pathogens were combined to comprise a mixed culture cocktail.

Sample preparation and inoculation. Spinach and tomatoes were purchased from a local market (Seoul, South Korea), washed in running water, then dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h before experiments to remove surface moisture. Spinach leaves and tomato surfaces were cut into 5 × 2 cm pieces. Type 304 stainless steel (SS) with No.4 finish (Ian industry, Ansan-si, South Korea) and glass (Corning Inc., NY, USA) were cut into coupons (5 × 2 cm), immersed in 70% ethanol for 20 min, and rinsed with distilled water. After washing, coupons

were dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h to remove surface moisture. Each coupon was used only once in order to avoid any surface changes.

Prepared samples were placed on aluminum foil in a laminar flow hood, and 0.1 ml of culture cocktail was inoculated onto one side of the sample by depositing droplets with a micropipettor at 14-16 locations. After inoculation, samples were dried in the laminar flow biosafety hood for 1 h at 22 ± 2 ℃.

ClO_2 gas treatment. ClO_2 gas treatment was conducted in a treatment system described previously (Park and Kang, 2015). Inoculated samples were placed in the treatment chamber with the inoculated surfaces facing upwards and covered with a plastic lid. Samples were treated with 20 ppmv ClO_2 gas for up to 30 min. The RH of the treatment chamber was adjusted to 92 ± 1% at 15 ± 0.5 ℃ (AH 11.4-12.3 g/m^3) and 51 ± 1% at 25 ± 0.5 ℃ (AH 11.2-12.3 g/m^3). When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and samples were exposed to the treatment. A thermohygrometer (SE-342, Center Technology Corp., Taiwan) was used to measure RH and temperature in the treatment chamber.

Absolute humidity was calculated from the RH and temperature data observed (Seinfeld and Pandis, 2006; Huang and Gustin, 2015):

$$\mathrm{AH} = \frac{P^0_{H_2O} \times \frac{RH}{100} \times 18\frac{g}{mol}}{R \times T}$$

where

$$\mathrm{P}^0_{H_2O} = 1013.25exp(13.3185A - 1.92A^2 - 0.6445A^3 - 0.1299A^4)$$

$$A = 1 - \frac{373.15}{T}$$

Where AH is absolute humidity (g/m^3), RH is relative humidity, R is the ideal gas law constant, and T is absolute temperature in degrees Kelvin.

Bacterial enumeration. Treated spinach leaves and tomatoes were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 30 ml of neutralizing buffer (Difco). Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. Treated stainless steel No.4 (SS No.4) and glass coupons were transferred to sterile 50-ml conical centrifuge tubes containing 30 ml of neutralizing buffer and 3 g of glass beads (425-600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then vortexed for 2 min. After homogenization, 1 ml aliquots withdrawn from stomacher bags or 50-ml conical centrifuge tubes were tenfold serially diluted in BPW, and 0.1 ml of appropriate diluents were spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Where low levels of surviving cells were expected, 1 ml aliquots withdrawn from stomacher bags were divided between four plates of each medium and spread-plated to lower the detection limit. The plates were incubated at 37 ℃ for 24–48 h. Colonies were enumerated after incubation and counts expressed as log CFU/cm^2.

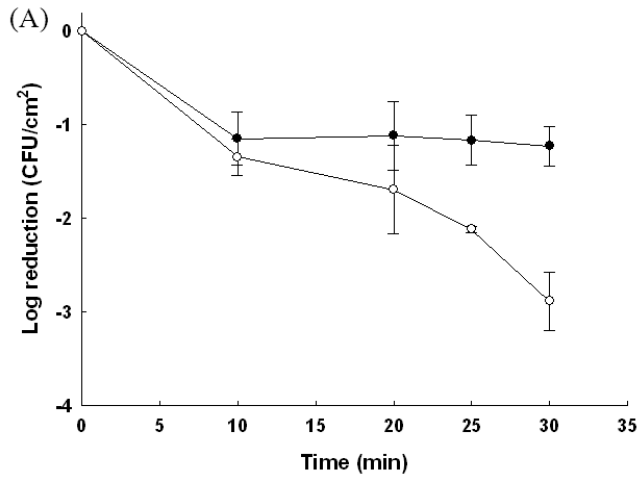
ClO_2 concentration on sample surfaces after treatment. Spinach leaves, tomatoes, and SS No.4 coupons were exposed to 20 ppmv of ClO_2 gas for 30 min under the same treatment conditions described above. Glass was exposed to the same conditions for 15 min. After treatment, spinach leaves (5 × 2 cm), whole tomatoes, and SS No.4 and glass coupons (5 × 2 cm) were immediately rinsed with 100 ml of sterile distilled water in sterile stomacher bags and massaged by hand for 5 min. Ten milliliters of sample were removed from each stomacher bag and tested by the DPD (N, N-diethyl-p-phenylenediamine) method using a Hach DR/820 Colorimeter (Hach, Loveland, CO) (Trinetta et al., 2011). The limit of detection for this method is 0.04 mg/L. ClO_2 concentration in rinse water were reported as mg/L. These experiments were conducted in triplicate.

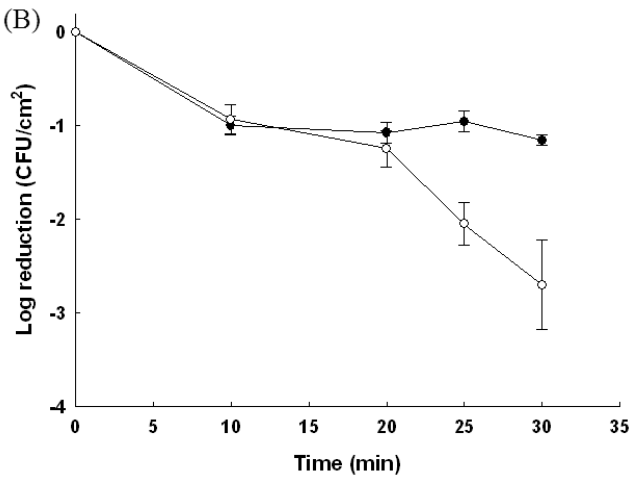
Statistical analysis. All experiments were repeated three times. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

II-3.3. Results

ClO_2 gas inactivation of foodborne pathogens. Figs. II-4 to 7 show log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach, tomatoes, SS No.4, and glass surfaces after treatment with 20 ppmv ClO_2 gas at 15 and 25 ℃. As treatment time increased, significant differences ($p < 0.05$) were observed between inactivation levels under different temperature conditions. ClO_2 gas treatment at 15 ℃ caused significantly more ($p < 0.05$) inactivation of the three pathogens than ClO_2 gas treatment at 25 ℃. Treatment with ClO_2 gas at 25 and 15 ℃ for 30 min resulted in 1.23, 1.15, and 1.54 log reductions, and 2.88, 2.70, and 2.53 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, on spinach (Fig. II-4). Exposure to ClO_2 gas at 25 and 15 ℃ for 30 min caused 1.73, 1.53, and 1.88 log reductions and 3.23, 2.82, and 2.93 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, on tomatoes (Fig. II-5). Levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on SS No.4 were reduced by 1.05, 1.00, and 1.78 log and 2.66, 2.37, and 3.03 log after ClO_2 gas treatment at 25 and 15 ℃ for 30 min, respectively (Fig. II-6). Treatment with ClO_2 gas at 25 ℃ for 20 min resulted in 2.24, 2.31, and 1.88 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on glass, respectively (Fig. II-7). The levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on

glass were reduced to below the detection limit (0.48 log CFU/cm^2) within 15 min when treated with ClO_2 gas at 15 ℃.





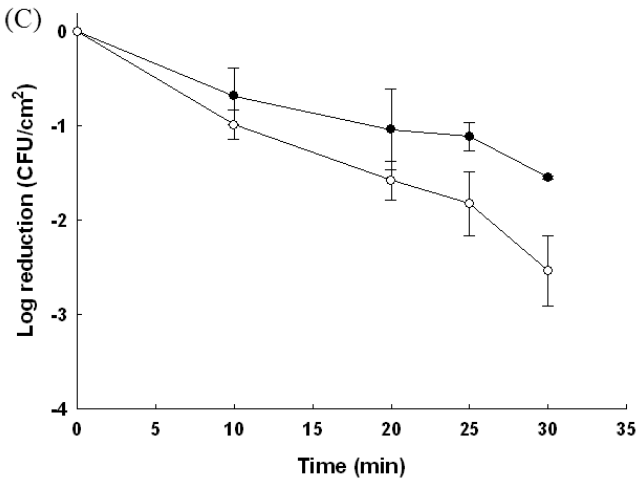


Fig. II-4. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on tomatoes treated with 20 ppmv ClO_2 gas at different temperatures. ●, 25 ℃; ○, 15 ℃. Values are expressed as the mean values of triplicate experiments, with error bars.

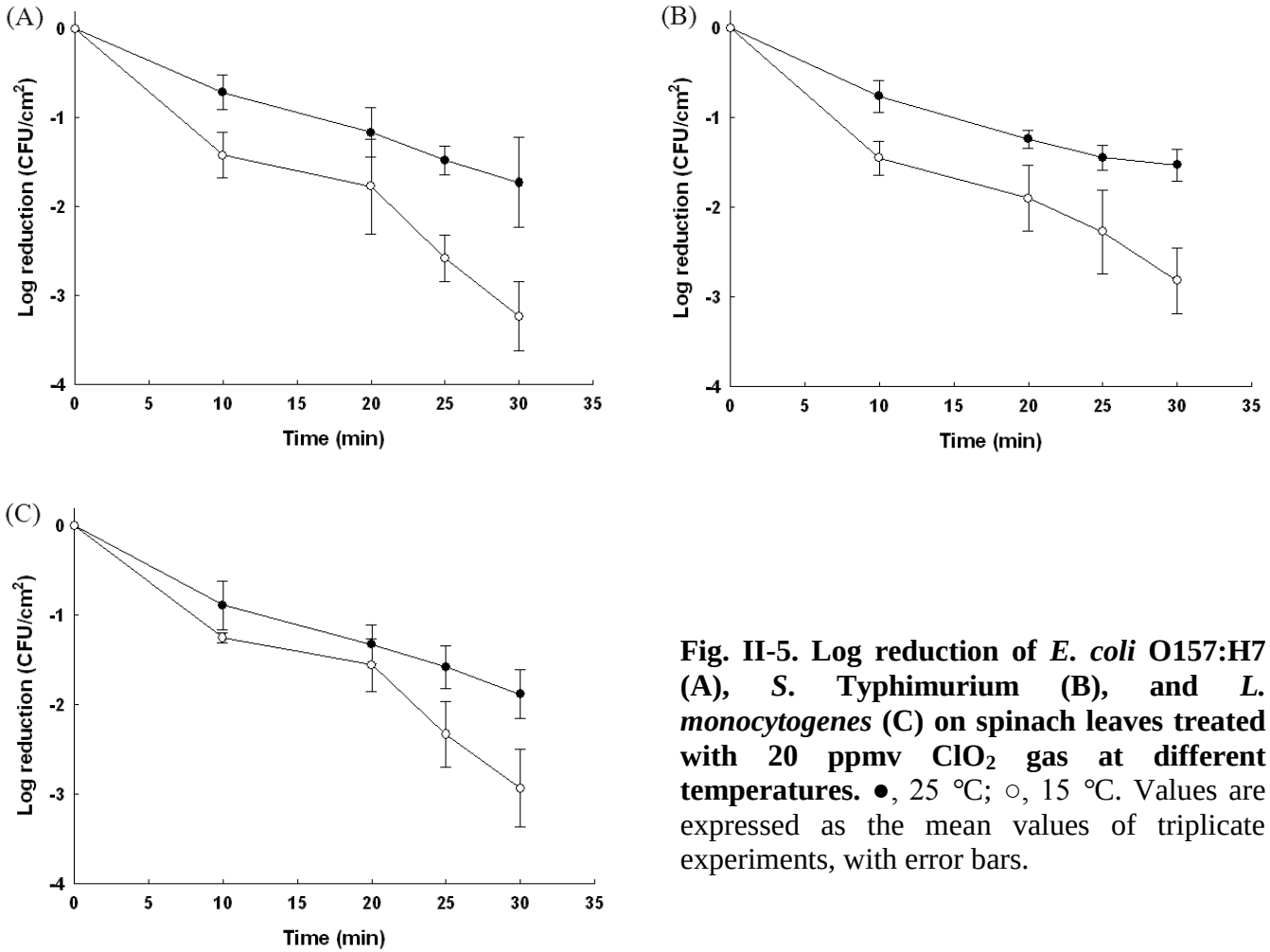


Fig. II-5. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on spinach leaves treated with 20 ppmv ClO_2 gas at different temperatures. ●, 25 ℃; ○, 15 ℃. Values are expressed as the mean values of triplicate experiments, with error bars.

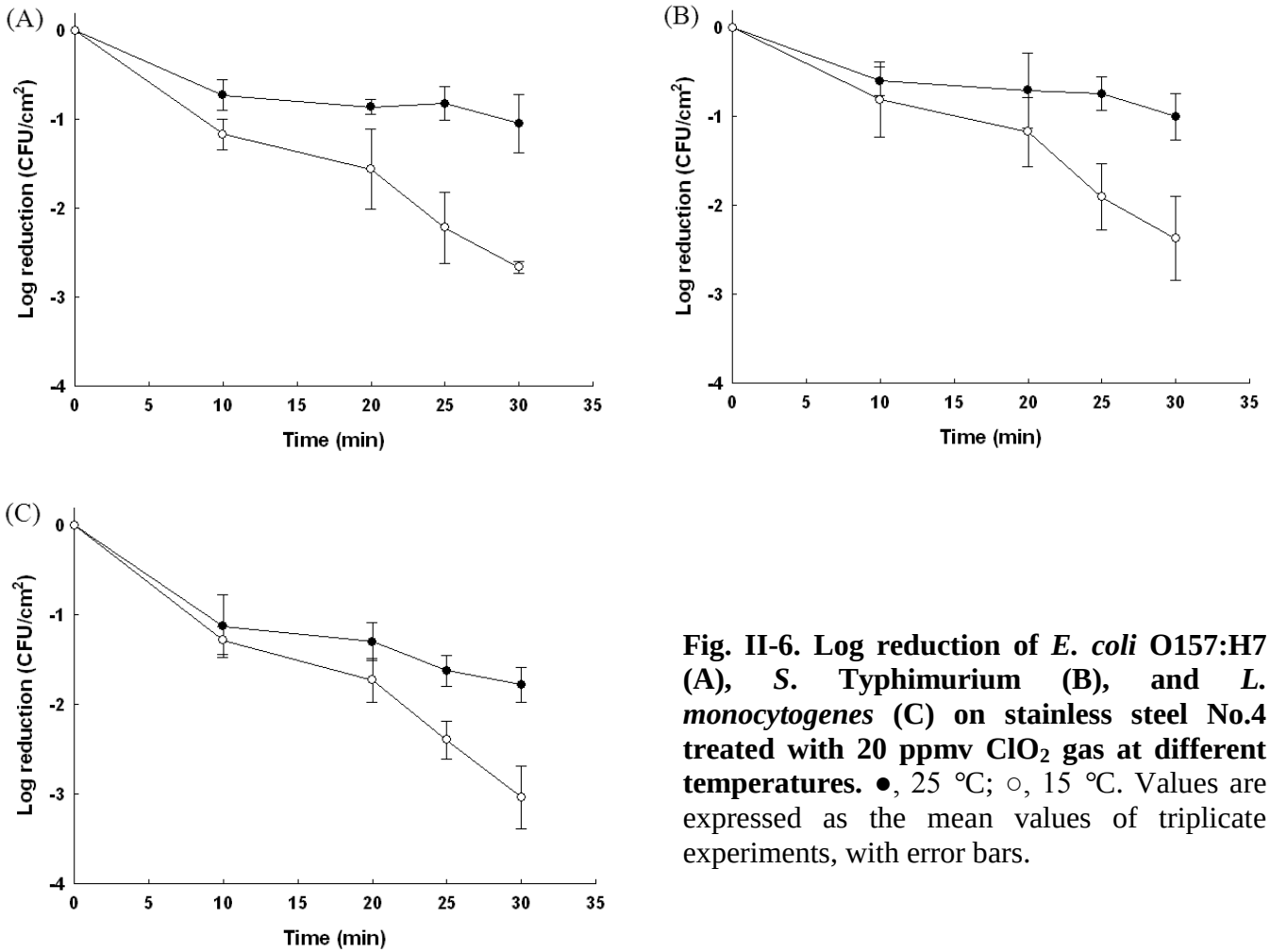


Fig. II-6. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on stainless steel No.4 treated with 20 ppmv ClO_2 gas at different temperatures. ●, 25 ℃; ○, 15 ℃. Values are expressed as the mean values of triplicate experiments, with error bars.

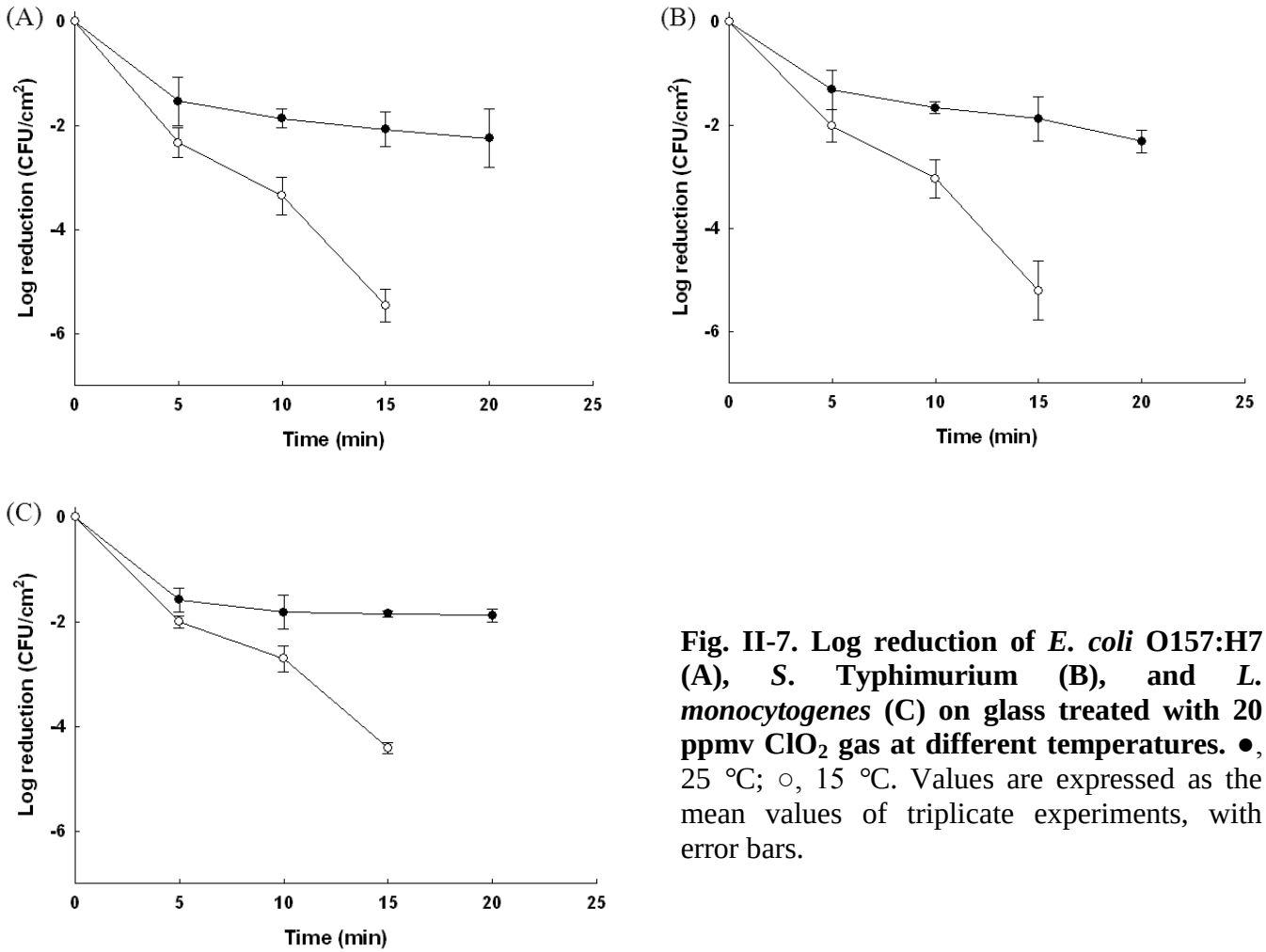


Fig. II-7. Log reduction of *E. coli* O157:H7 (A), *S.* Typhimurium (B), and *L. monocytogenes* (C) on glass treated with 20 ppmv ClO_2 gas at different temperatures. ●, 25 ℃; ○, 15 ℃. Values are expressed as the mean values of triplicate experiments, with error bars.

ClO_2 concentration on sample surfaces after treatment. Fig. II-8 shows levels of ClO_2 concentration on each type of sample treated with ClO_2 gas at 15 and 25 ℃. ClO_2 concentrations after gas treatment at 15 ℃ were significantly ($p < 0.05$) higher than those treated at 25 ℃. After ClO_2 gas treatment at 25 ℃ for 30 min, ClO_2 concentrations on spinach, tomatoes, and SS No.4 were 0.04, 0.06, and 0.01 mg/L, respectively. ClO_2 concentrations on these same samples after exposure to ClO_2 gas at 15 ℃ for 30 min were 0.07, 0.09, and 0.05 mg/L, respectively. After 15 min treatment with ClO_2 gas at 25 and 15 ℃, ClO_2 concentrations on glass were 0.07 and 0.10 mg/L, respectively.

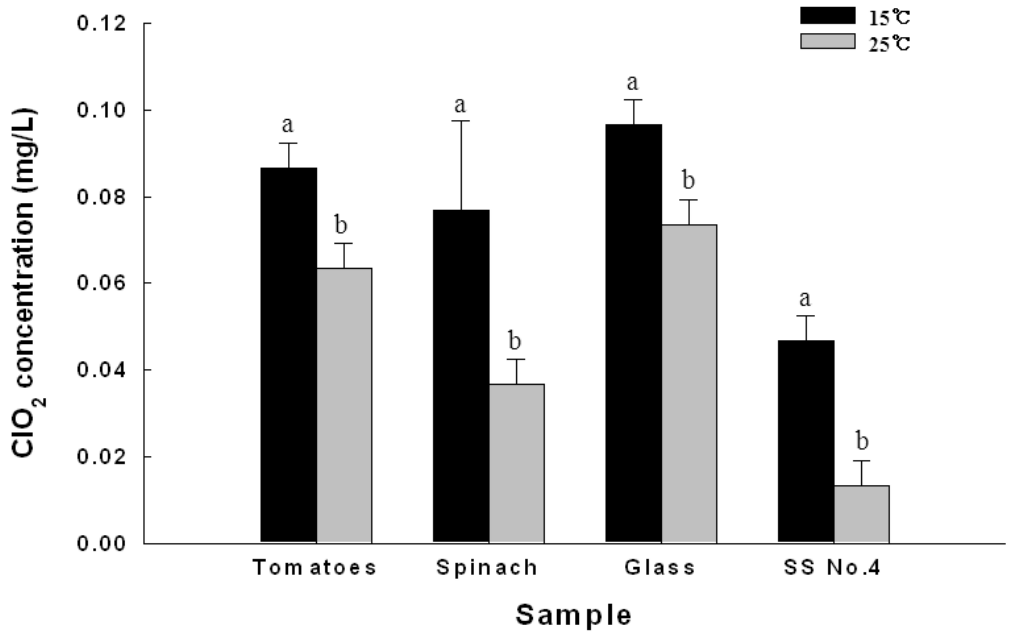


Fig. II-8. ClO_2 concentration on sample surfaces immediately after treatments with 20 ppmv ClO_2 gas under different temperatures. Values with different lowercase letters were significantly different ($p < 0.05$). Values are expressed as the mean values of triplicate experiments, with error bars.

II-3.4. Discussion

It is known that ClO_2 gas acts similar to aqueous ClO_2 for inactivating microorganisms due to its high solubility in water (Linton et al., 2006). As is the case with other gases, the solubility of ClO_2 increases as temperature decreases. The mole fraction solubility of ClO_2 (at 101.325 kPa partial pressure of gas) is 2.67×10^{-2}, 1.82×10^{-2}, and 1.26×10^{-2} at 15, 25, and 35 ℃, respectively (Haller and Northgraves, 1955; Kepinski and Trzeszczynski, 1964). Temperature may also affect the reactivity of sanitizers. Generally, inactivation efficacy of aqueous sanitizers increases with temperature across a moderate temperature range. Aqueous ClO_2 was more effective at reducing *E. coli* O157:H7 and *Pseudomonas aeruginosa* at 20 ℃ than at 10 ℃ (Taylor et al., 1999). Increasing temperature within the range of 5-30 ℃ led to increased inactivation of *Mycobacterium avium* by aqueous ClO_2 (Vicuña-Reyes et al., 2008). In the case of ozone, Larson and Mariñas (2003) reported increased rates of ozone inactivation of *Bacillus subtilis* spores with increasing temperature within a range of 1–30 ℃. Increasing temperature within a range 7-22 ℃ strongly influenced the inactivation rate of *B. subtilis* spores in oxidant demand-free phosphate buffer and also led to a decrease in the CT 2 log reduction (Dow et al., 2006). Steenstrup and Floros (2004) reported that high temperatures resulted in lower D-values and shorter lag times when apple cider was treated with ozone.

Based on these studies, there is no consensus regarding the effect of temperature on the antimicrobial efficacy of ClO_2 gas, because reactivity of ClO_2 gas increases but ClO_2 gas becomes less soluble in water as temperature increases. The simultaneous effect of these two factors (reactivity and solubility) on the antimicrobial efficacy of ClO_2 gas could vary with experimental conditions. In the present study, higher microbial inactivations were observed at 15 rather than 25 ℃. Also, ClO_2 concentration on sample surfaces after ClO_2 gas treatment at 15 ℃ were significantly ($p < 0.05$) higher than those treated at 25 ℃. These results indicate that ClO_2 solubility rather than reactivity has greater influence on the antimicrobial effect of ClO_2 gas. Although the reactivity of ClO_2 gas decreases with reduced temperature, increasing solubilized ClO_2 on sample surfaces may cause greater reductions of foodborne pathogens.

Han et al. (2001a) evaluated the effects and interactions of ClO_2 gas concentration, RH, treatment time, and temperature on inactivation of *E. coli* O157:H7, and reported that temperature was the least significant factor influencing inactivation of *E. coli* O157:H7. However, in that study, it was difficult to determine the effect of humidity on microbial inactivation according to variations of temperature, because they used RH rather than AH as a humidity parameter. RH is the ratio between the actual water vapor pressure of the air and the water vapor pressure of saturated air at a certain temperature, and AH is the actual water content of the air (Shaman and Kohn, 2009; Zhao et al., 2012). RH varies as a function of

the temperature or the actual water vapor content of air because the water vapor pressure of saturated air increases exponentially as temperature increases, while AH is irrespective of temperature (Shaman and Kohn). Therefore, experiments should be performed under the same AH conditions to evaluate the effect of temperature on microbial inactivation by ClO_2 gas.

In conclusion, this study showed that treatment temperature has a great impact on the inactivation of foodborne pathogens by ClO_2 gas treatment. The results of this study indicate that increased solubility of ClO_2 gas at low temperatures plays an important role in bacterial inactivation from surfaces. The results of this study can help the food industry establish optimized ClO_2 gas treatment conditions.

Chapter III.

Combination treatments of ClO_2 gas with various sanitizing technologies

III-1. Inactivation of foodborne pathogens on produce by combined treatment with ClO_2 gas and UV-C radiation, and mechanisms of synergistic inactivation

III-1.1. Introduction

The consumption of fresh produce has significantly increased due to heightened public awareness of the importance of healthy eating (Dikici et al., 2015). However, with increasing produce consumption, the number of produce-related foodborne outbreaks has also increased (Lynch et al., 2009). Fresh produce was implicated in almost 23% of all foodborne illnesses from 1998 to 2007 (DeWaal et al., 2009). Spinach and spinach-containing products were associated with an outbreak of *Escherichia coli* O157:H7 (CDC, 2006; Maki 2006) which infected a total of 205 persons and resulted in 4 deaths (Wendel et al., 2009). A total of 33 persons infected with *E. coli* O157:H7 traced to organic spinach and spring mix blend was reported from 5 US states in 2012 (CDC, 2012). Tomatoes were associated with more than 14 outbreaks of foodborne illness between 1996 and 2008, and approximately 1,990 human culture-confirmed cases of salmonellosis that occurred during 1990-2007 were traced to various types of tomatoes (CDC, 2007; Gravani, 2009).

Chlorine dioxide (ClO_2) has emerged as a promising non-thermal sanitizing technology for fresh produce in recent years (Bhagat et al., 2010). ClO_2 is a strong oxidizing agent with a broad antimicrobial spectrum and its efficacy is not greatly affected by pH or presence of organic matter (Beuchat, 1998, Trinetta et al., 2012). ClO_2 gas has been evaluated for inactivating foodborne pathogens on fresh produce such as spinach (Neal et al., 2012), potatoes (Wu and Rioux, 2010), apples (Du et al.,

2002), tomatoes (Bhagat et al., 2010, Trinetta et al., 2013), lettuce (Mahmoud and Linton, 2008), mung bean sprouts (Prodduk et al., 2014), cabbage (Sy et al., 2005), cantaloupe (Mahmoud et al., 2008), and strawberries (Han et al., 2004). However, the concentration of ClO_2 gas used in previous studies was excessive (Morino et al., 2011).

Combinations of different technologies, known as hurdle technology, could be an alternative to the use of high ClO_2 gas concentrations. Combined treatments can achieve required levels of food safety and the maintenance of organoleptic qualities of foods, while decreasing the intensity of each hurdle, that is, the antimicrobial concentration (Leistner and Gorris, 1995). Ultraviolet (UV) radiation, another non-thermal technology, has been approved for use as a disinfectant for surface treatment of foods (FDA, 2002). Since it can cause cumulative damage to microbial DNA, UV radiation was recommended for use in combination with other techniques (Rame et al., 1997). Antimicrobial effects of the combination of UV radiation with chemical agents such as hydrogen peroxide (Hadjok et al., 2008), ozone (Selma et al., 2008), and sodium hypochlorite (Ha and Ha, 2011) have been reported. However, none of the studies examined the antimicrobial effect of UV-C radiation (UVC) in combination with ClO_2 gas.

The objective of this study was to evaluate the antimicrobial effects of ClO_2 gas combined with UVC against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach leaves and tomatoes. The mechanism of

inactivation was investigated by measuring leakage of UV-absorbing substances and analyzing transmission electron microscopy. Also, any changes in color and texture of samples were assessed.

III-1.2. Materials and Methods

Bacterial strains and cell suspension. Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea), for this study. All strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were cultured individually in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 ℃ for 24 h, followed by centrifugation at 4000 × *g* at 4 ℃ for 20 min, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in BPW, corresponding to approximately 10^7-10^8 CFU/ml. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* strains were combined to make culture cocktails for use in experiments.

Sample preparation and inoculation. Spinach and whole tomatoes were purchased from a local market (Seoul, South Korea). These products were washed in running water and dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h before experiments. Spinach leaves were trimmed to approximately 5 × 3 cm in size, and the outer surface of tomatoes was cut into 5 × 2 cm pieces. Prepared spinach leaves and tomato surface samples were placed on aluminum foil in a laminar flow biosafety hood, and 0.1 ml of culture cocktail was inoculated onto one side of each

prepared sample by depositing droplets with a micropipettor at 15–20 locations. After inoculation, samples were dried in the hood for 1 h at 22 ± 2 ℃ with the fan running.

Combined treatment of UVC and ClO_2 gas. The combined treatment of UVC and ClO_2 gas was conducted in a treatment system described previously with slight modification (Park and Kang, 2015). A germicidal UV lamp (G6T5, Sankyo, Japan) with a nominal output power of 6 W was used as a UVC emitting source. The UV lamp was located in the ceiling of the treatment chamber and was allowed to stabilize by turning it on for at least 5 min before experiments.

Procedures for treating samples. Inoculated spinach leaves and tomatoes were placed in the treatment chamber with the inoculated surfaces facing upwards and covered with a plastic lid. For UVC treatment alone, samples were treated with UVC for 20 min (radiation intensity, 70.68μW/cm^2 at the sample location). The UVC (at 253.7 nm wavelength) intensity was measured by using a spectrometer (AvaSpec-ULS2048-USB2-UA-50, Avantes, Netherlands). For ClO_2 gas treatments alone, samples were treated with 5 or 10 ppmv ClO_2 gas for 20 min. For combined treatments, samples were subjected with simultaneous treatment of UVC and ClO_2 gas (5 or 10 ppmv) for 20 min. All experiments were performed at 22 ± 1 ℃, and RH of the treatment chamber was adjusted with distilled water to 90 ± 2% during treatment. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed. Samples were withdrawn from the treatment chamber after

5, 10, 15, and 20 min exposure to each treatment, and treated samples were used to determine surviving bacterial populations. These experiments were repeated three times.

Bacterial enumeration. Treated spinach leaves (5 ± 0.2 g) and one piece of tomato were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 45 and 30 ml of neutralizing buffer (Difco), respectively. Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. After homogenization, 1 ml aliquots of the sample were tenfold serially diluted in 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Where low numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates to lower the detection limit. The plates were incubated at 37 ℃ for 24–48 h. After incubation, colonies were counted and calculated as log CFU/g for spinach leaves and log CFU/cm^2 for tomatoes, respectively.

Leakage of UV-absorbing substances. Each cell suspension (1 ml) of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was inoculated onto glass petri dishes, and dried in the hood for 1 h at 22 ± 2 ℃ with the fan running. Each

pathogen inoculated onto glass petri dishes was treated with UVC, ClO_2 gas (10 ppmv), and the combined treatment of UVC and ClO_2 gas (UVC-ClO_2) for 15 min: previously confirmed that inactivation patterns of three pathogens on glass petri dishes were similar with those on tomato surfaces. Treated cells were resuspended using 10 ml of phosphate-buffered saline (PBS; pH 7.0), and centrifuged at 10,000 × *g* at 4 ℃ for 10 min. The upper 1 ml of the supernatant was removed, and the UV absorbance was measured at a wavelength of 260 and 280 nm with a spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA). The absorbance was presented as the mean of triplicated measurements.

Transmission electron microscopy analysis. Transmission electron microscopy (TEM) analysis was conducted after UVC, ClO_2 gas (10 ppmv), and UVC-ClO_2 gas (10 ppmv) treatment for 15 min to investigate structural damages of pathogen cells. Treated *S.* Typhimurium cells on glass petri dishes described above were resuspended using 10 ml of PBS and collected by centrifugation at 4,000 × *g* at 4 ℃ for 10 min. The cells were fixed at 4 ℃ for 4 h in modified Karnovsky's fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After primary fixation, each sample was centrifuged and washed three times with 0.05 M sodium cacodylate buffer (pH 7.2) at 4 ℃ for 10 min. Cells were postfixed with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 ℃ for 2 h and briefly washed twice with distilled water at room temperature. The washed cells were stained overnight with 0.5% uranyl acetate at

4 ℃. The cells were then dehydrated at room temperature using a graded ethanol series (10 min each in 30, 50, 60, 70, 95, and 100%), finishing with three consecutive 100% ethanol washes. The transition was performed with 100% propylene oxide at room temperature for 15 min. The cells were then infiltrated for 2 h with a 1:1 solution of propylene oxide and Spurr's resin, and then placed in Spurr's resin overnight. In order to get specimen blocks, the polymerization of the resin was conducted in an oven at 70 ℃ for 24 h. Specimens were sectioned (70-nm thick) by means of an ultramicrotome (MT-X; RMC, Tucson, AZ, USA) and then stained with 2% uranyl acetate for 7 min, followed by Reynolds' lead citrate for 7 min. The sections were then observed with a transmission electron microscope (Libra 120; Carl Zeiss, Heidenheim, Germany).

Measurement of color and texture of samples. After combined treated with UVC and ClO_2 gas (10 ppmv), uninoculated spinach leaves and tomatoes were stored at 4 and 12 ℃ for 7 days, respectively, to identify quality changes during storage following treatments. Hunter's L, a, b values of the sample were measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each sample. The texture of spinach leaves and tomatoes was measured with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set and cylinder probe with a 4 mm diameter, respectively. Twenty grams of spinach leaves was placed onto the press holder with the stems positioned perpendicular to the path of the blade, and a blade was moved down at 2

mm/s (path length 10 mm). For tomatoes, the loading rate and path length were also set at 2 mm/s and 10 mm. Maximum force (N) was recorded using Texturepro CT software (Brookfield Engineering Laboratories, Inc.). Three measurements were performed for each treatment with independently-prepared samples.

Statistical analysis. All experiments were done in triplicate. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

III-1.3. Results

Effects of UVC, ClO_2 gas, and UVC-ClO_2 gas treatments on populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. The reduction in numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves during UVC, ClO_2 gas, and simultaneous application of both technologies is presented in Tables III-1 and 2. Generally, antimicrobial effects of UVC-ClO_2 gas (5 ppmv) treatment were not superior to those of individual treatments during 15 min. After 20 min treatment, UVC-ClO_2 gas (5 ppmv) treatment showed the additive effect: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from the sum of individual treatments. Treatment with UVC for 20 min caused 1.85, 2.02, and 1.87 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Exposure to 5 ppmv of ClO_2 gas for 20 min resulted in 2.19, 2.17, and 1.58 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. UVC-ClO_2 gas (5 ppmv) treatment resulted in 4.38, 3.73, and 3.14 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

Similarly, UVC-ClO_2 gas (10 ppmv) treatment showed additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after 20 min treatment. Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 3.56, 3.61, and 3.23 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*,

respectively. UVC-ClO_2 gas (10 ppmv) treatment caused 5.17, 5.41, and 4.32 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

UVC-ClO_2 gas (5 ppmv) treatment showed clear additive effects on tomatoes earlier than on spinach leaves (Table III-3). Most of UVC-ClO_2 gas (5 ppm) treatments showed a more significant reduction than that of each treatment applied individually following 5 min treatment. After 15 min treatment, UVC-ClO_2 gas (5 ppmv) treatment produced an additive effect in inactivating *E. coli* O157:H7 and *S.* Typhimurium. Treatment with UVC for 20 min caused 2.02, 1.96 and 1.58 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells were reduced by 20 min treatment of ClO_2 gas (5 ppmv), showing 2.34, 2.24, and 1.57 log reductions, respectively. UVC-ClO_2 gas (5 ppmv) treatment resulted in 4.80, 4.28, and 2.70 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

Synergistic effects in inactivating *E. coli* O157:H7 and *S.* Typhimurium were observed after UVC-ClO_2 gas (10 ppmv) treatment for 15 min (Table III-4). UVC-ClO_2 gas (10 ppmv) treatment for 15 min achieved 5.62 and 5.46 log reductions in *E. coli* O157:H7 and *S.* Typhimurium, respectively. For both pathogens, UVC-ClO_2 gas (10 ppmv) treatment produced a more significant ($p < 0.05$) reduction than the sum of UVC and ClO_2 gas (10 ppmv) inactivation after exposure times of 15 min or

more. In case of *L. monocytogenes*, the synergistic effect was observed after UVC-ClO_2 gas (10 ppmv) treatment for 20 min.

Table III-1. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with UV-C radiation (UVC), 5 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Bacteria	Treatment	Log reduction (log CFU/g)			
		5 min	10 min	15 min	20 min
E. coli O157:H7	UVC	1.16±0.01Aa[b]	1.53±0.17ABa	1.69±0.31ABa	1.85±0.49Ba
	ClO_2 gas	1.34±0.24Aa	1.86±0.40ABa	1.95±0.23ABa	2.19±0.39Ba
	UVC-ClO_2 gas	1.94±0.19Ab	2.29±0.55Aa	2.46±0.20Ab	4.38±0.19Bb
S. Typhimurium	UVC	1.35±0.13Aa	1.56±0.07ABa	2.01±0.26Ba	2.02±0.42Ba
	ClO_2 gas	1.09±0.22Aa	1.57±0.14Ba	1.76±0.30BCa	2.17±0.26Ca
	UVC-ClO_2 gas	1.67±0.02Ab	1.78±0.08Ab	2.25±0.14Ba	3.73±0.04Cb
L. monocytogenes	UVC	1.13±0.43Aa	1.54±0.46Aa	1.71±0.49Aab	1.87±0.50Aa
	ClO_2 gas	0.74±0.35Aa	0.82±0.35Aa	1.26±0.33ABa	1.58±0.49Ba
	UVC-ClO_2 gas	1.44±0.38Aa	1.63±0.54Aa	2.13±0.40Ab	3.14±0.25Bb

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-2. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with UV-C radiation (UVC), 10 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Bacteria	Treatment	Log reduction (log CFU/g)			
		5 min	10 min	15 min	20 min
E. coli O157:H7	UVC	1.32±0.15Aa[b]	1.69±0.01ABa	1.85±0.47ABa	2.01±0.33Ba
	ClO_2 gas	1.15±0.19Aa	2.44±0.26Bb	3.01±0.34Cb	3.56±0.34Db
	UVC-ClO_2 gas	2.29±0.06Ab	3.16±0.25Bc	3.73±0.48Cb	5.17±0.23Dc
S. Typhimurium	UVC	1.44±0.02Aa	1.65±0.08ABa	2.10±0.13Ba	2.10±0.45Ba
	ClO_2 gas	1.36±0.19Aa	2.37±0.12Bb	2.99±0.28Cb	3.61±0.46Db
	UVC-ClO_2 gas	1.72±0.06Ab	2.22±0.16Bb	3.36±0.05Cc	5.47±0.32Dc
L. monocytogenes	UVC	0.96±0.28Aa	1.37±0.31ABa	1.55±0.36ABa	1.70±0.38Ba
	ClO_2 gas	0.56±0.28Aa	1.61±0.43Ba	2.38±0.38Cb	3.23±0.35Db
	UVC-ClO_2 gas	1.66±0.41Ab	2.36±0.13Bb	2.96±0.31Bb	4.32±0.52Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-3. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes treated with UV-C radiation (UVC), 5 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Bacteria	Treatment	Log reduction (log CFU/cm^2)			
		5 min	10 min	15 min	20 min
E. coli O157:H7	UVC	1.44±0.15Aa[b]	1.73±0.46Aa	1.80±0.30Aa	2.02±0.13Aa
	ClO_2 gas	1.27±0.33Aa	1.61±0.18ABa	1.92±0.17BCa	2.34±0.18Cb
	UVC-ClO_2 gas	2.21±0.36Ab	2.57±0.15Ab	4.31±0.10Bb	4.80±0.14Cc
S. Typhimurium	UVC	1.48±0.27Ab	1.62±0.32ABa	1.93±0.23ABa	1.96±0.07Ba
	ClO_2 gas	1.04±0.03Aa	1.20±0.22ABa	1.62±0.50Ba	2.24±0.27Ca
	UVC-ClO_2 gas	1.92±0.23Ac	2.63±0.23Bb	3.38±0.23Cb	4.28±0.13Db
L. monocytogenes	UVC	0.78±0.38Aa	1.18±0.30ABa	1.48±0.08Ba	1.58±0.10Ba
	ClO_2 gas	0.86±0.13Aa	1.04±0.35ABa	1.36±0.11BCa	1.57±0.15Ca
	UVC-ClO_2 gas	1.38±0.51Aa	1.52±0.31Bb	2.36±0.06Cb	2.98±0.31Db

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-4. Log reductions[a] of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes treated with UV-C radiation (UVC), 10 ppmv ClO_2 gas, and both technologies simultaneously (UVC-ClO_2 gas).

Bacteria	Treatment	Log reduction (log CFU/cm^2)			
		5 min	10 min	15 min	20 min
E. coli O157:H7	UVC	1.69±0.30Aa[b]	1.85±0.34ABa	2.05±0.06ABa	2.27±0.15Ba
	ClO_2 gas	1.63±0.28Aa	2.42±0.17Ba	2.72±0.04Bb	3.81±0.05Cb
	UVC-ClO_2 gas	1.85±0.09Aa	3.86±0.54Bb	5.62±0.27Cc	> 6.79Dc
S. Typhimurium	UVC	1.71±0.06Ab	1.85±0.20Aa	2.16±0.38Aa	2.20±0.24Aa
	ClO_2 gas	1.20±0.06Aa	1.76±0.23Ba	2.58±0.37Ca	3.85±0.37Db
	UVC-ClO_2 gas	2.00±0.26Ab	3.28±0.53Bb	5.46±0.51Cb	> 7.07Dc
L. monocytogenes	UVC	0.86±0.36Aa	1.26±0.42ABa	1.55±0.25Ba	1.66±0.14Ba
	ClO_2 gas	0.82±0.50Aa	1.38±0.47Aa	2.13±0.22Bb	3.13±0.16Cb
	UVC-ClO_2 gas	1.22±0.19Aa	1.91±0.08Ba	3.15±0.13Cc	> 6.33Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Leakage of UV-absorbing substances. Leakage of UV-absorbing substances from *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells measured at 260 nm is shown in Fig. III-1A, 2A, and 3A. The levels of UV-absorbing substances in ClO_2 gas (10 ppmv) and UVC-ClO_2 gas (10 ppmv) treated cells were much greater than those of UVC treated cells. Increasing the treatment time resulted in increased levels of UV-absorbing substances when they were treated with ClO_2 gas (10 ppmv) and UVC-ClO_2 gas (10 ppmv). Among them, leakage of UV-absorbing substances began to be remarkable after 15 min treatment of UVC-ClO_2 gas (10 ppmv). Leakage of UV-absorbing substances of *E. coli* O157:H7 and *S.* Typhimurium cells treated with UVC-ClO_2 gas (10 ppmv) was significantly ($p < 0.05$) higher than the sum of levels of UV-absorbing substances treated with UVC and ClO_2 gas (10 ppmv) after 15 min treatments. Similar patterns were observed in levels of UV-absorbing substances measured at 280 nm (Fig. III-1B, 2B, and 3B). Leakage of UV-absorbing substances of *S.* Typhimurium treated with UVC-ClO_2 gas (10 ppmv) was significantly ($p < 0.05$) higher than the sum of levels of UV-absorbing substances treated with UVC and ClO_2 gas (10 ppmv).

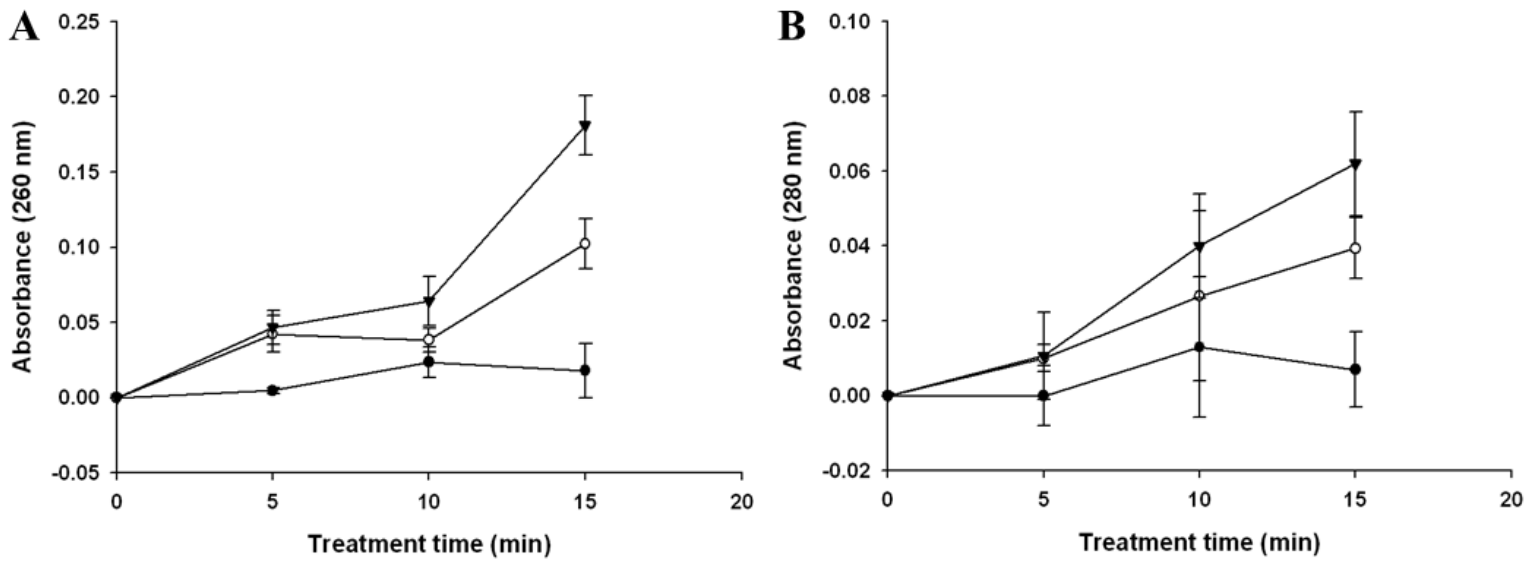


FIG. III-1. Leakage of UV-absorbing substances from *E. coli* O157:H7 cells treated with UVC, ClO_2 gas (10 ppmv), and UVC-ClO_2 gas as a function of treatment time. Symbols: ●, treated with UVC; ○, treated with ClO_2 gas (10 ppmv); ▼, treated with UVC-ClO_2 gas.

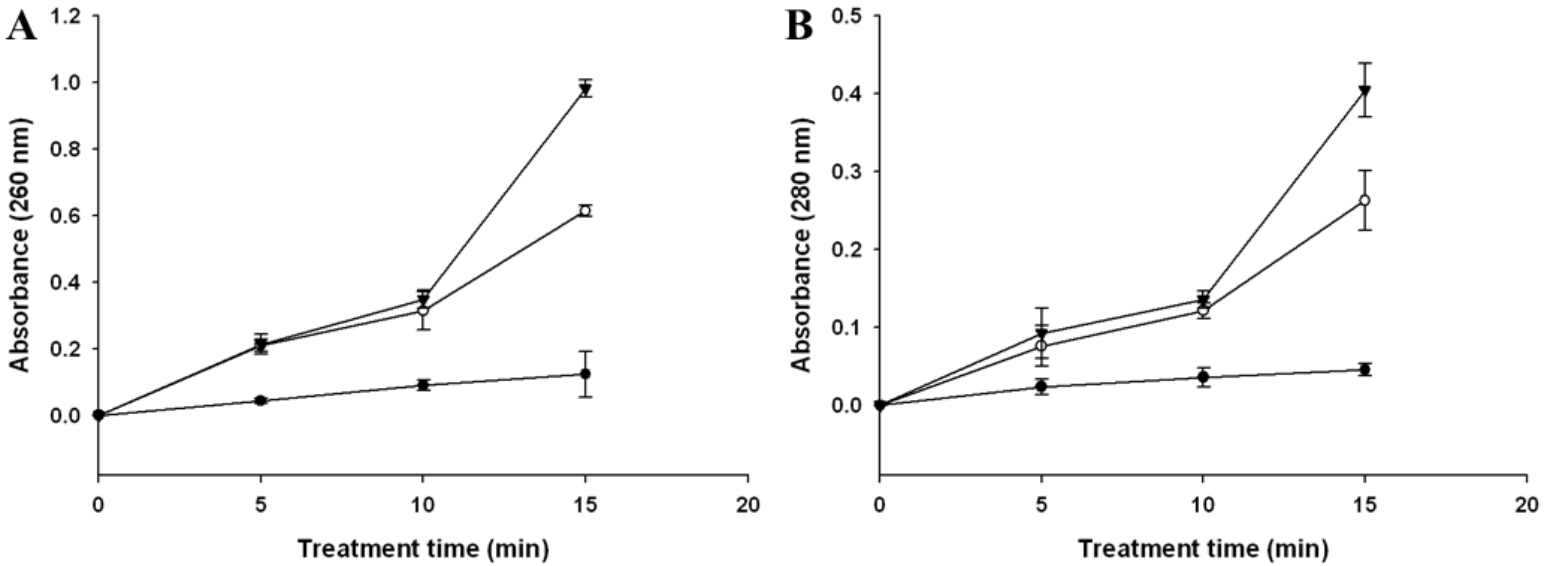


FIG. III-2. Leakage of UV-absorbing substances from *S.* Typhimurium cells treated with UVC, ClO_2 gas (10 ppmv), and UVC-ClO_2 gas as a function of treatment time. Symbols: ●, treated with UVC; ○, treated with ClO_2 gas (10 ppmv); ▼, treated with UVC-ClO_2 gas.

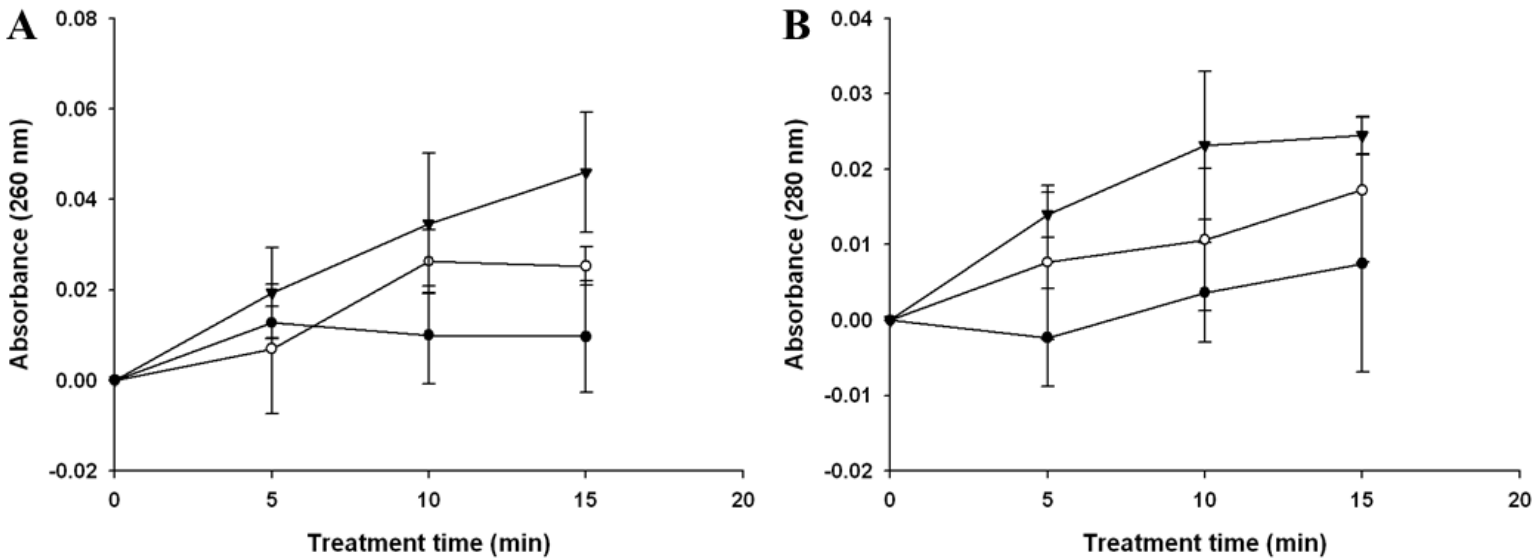


FIG. III-3. Leakage of UV-absorbing substances from *L. monocytogenes* cells treated with UVC, ClO_2 gas (10 ppmv), and UVC-ClO_2 gas as a function of treatment time. Symbols: ●, treated with UVC; ○, treated with ClO_2 gas (10 ppmv); ▼, treated with UVC-ClO_2 gas.

Transmission electron microscopy analysis. TEM images were used to analyze the morphological changes occurring in *S.* Typhimurium cells treated with UVC, ClO_2 gas (10 ppmv), and simultaneous application of both technologies, as seen in Fig. III-4. Individual treatments of *S.* Typhimurium cells with ClO_2 gas (10 ppmv) was shown to cause some changes (Fig. III-4C) compared to untreated cells (Fig. III-4A), such as the uneven distribution and aggregation of internal cellular substances. Also, slight separations of the cell membrane from cytoplasm were observed. These phenomena were much more pronounced in the case of *S.* Typhimurium cells treated with UVC-ClO_2 gas (10 ppmv) (Fig. III-4D). Severe rupturing of cell membranes and leakage of intracellular contents were observed.

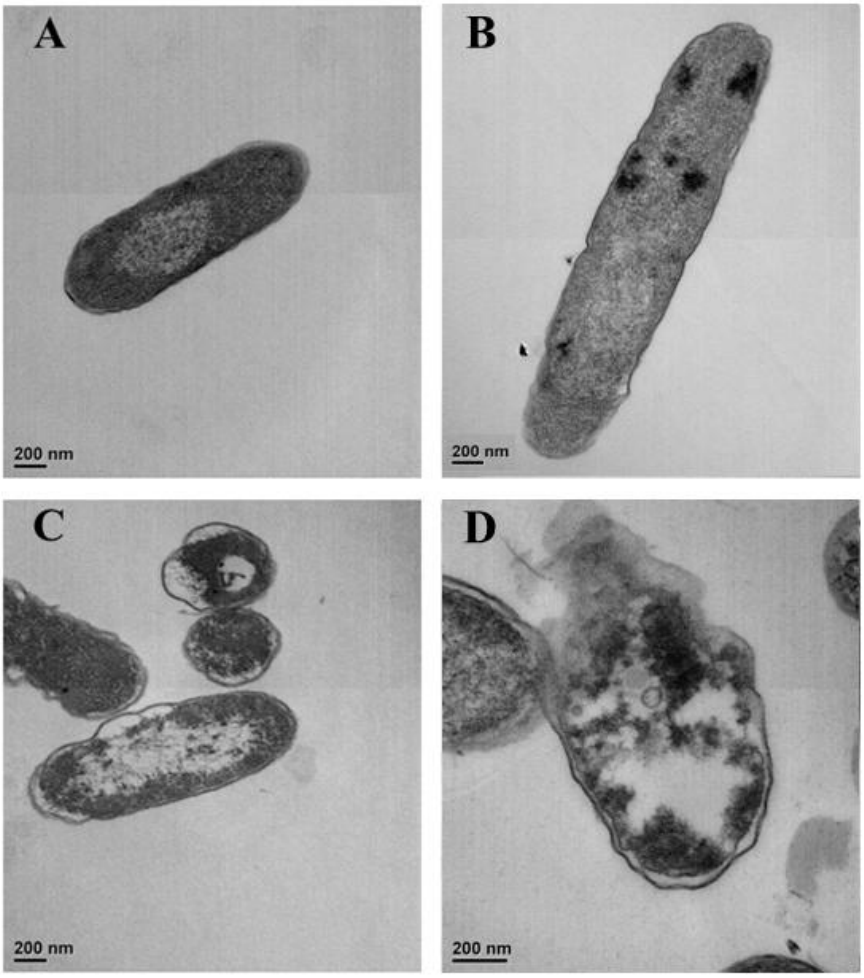


FIG. III-4. Transmission electron microscopy images of *S*. Typhimurium treated with UVC, ClO_2 gas, and UVC-ClO_2 gas for 15 min. (A), untreated cells; (B) UVC treated cells; (C), ClO_2 gas (10 ppmv) treated cells; (D), UVC-ClO_2 gas treated cells.

The quality changes of spinach leaves and tomatoes during storage. The *a** value of spinach leaves decreased while the *b** value increased during storage. However, there were no significant ($p > 0.05$) differences in Hunter's color values (*L**, *a**, *b**) between untreated samples (control) and those treated with UVC-ClO_2 gas (10 ppmv) during storage at 4 ℃ for 7 days (Table III-5). In the case of tomatoes, the *L** value decreased and the *b** values increased during storage. However, there were also no significant ($p > 0.05$) differences in Hunter's color values (*L**, *a**, *b**) between the control and those treated with UVC-ClO_2 gas (10 ppmv) during storage. Table III-6 shows the effects of UVC-ClO_2 gas (10 ppmv) treatment on the texture of spinach leaves and tomatoes. There were no significant ($p > 0.05$) differences in texture between control and treated samples during storage for 7 days.

Table III-5. Changes in color values[a] of spinach leaves and tomatoes treated in combination with UVC and ClO_2 gas (10 ppmv) during storage for 7 days.

Day	Treatment			
	Spinach leaves		Tomatoes	
	Control	UVC + ClO_2 gas	Control	UVC + ClO_2 gas
		*L**		
0	40.59±1.33A[b]	40.79±0.62A	43.84±0.73A	43.69±0.91A
2	40.60±1.10A	40.81±2.23A	44.72±1.21A	43.63±0.64A
4	40.56±1.06A	40.06±1.37A	42.59±0.53A	41.45±1.76A
7	39.60±1.56A	39.27±1.48A	40.85±1.33A	40.20±2.03A
		*a**		
0	-7.12±0.84A	-7.77±0.28A	18.42±2.13A	18.29±0.64A
2	-7.09±0.89A	-7.50±0.90A	19.79±2.37A	20.21±1.59A
4	-7.97±0.80A	-8.42±0.73A	19.34±1.23A	19.64±1.02A
7	-8.78±0.55A	-9.30±1.09A	20.47±0.87A	19.85±1.27A
		*b**		
0	9.45±1.03A	9.83±0.32A	19.74±1.56A	19.81±0.39A
2	9.20±1.23A	9.78±0.82A	24.48±0.55A	24.72±0.79A
4	10.57±1.59A	10.40±1.26A	25.16±1.95A	25.44±1.53A
7	12.79±1.60A	12.93±1.49A	24.41±0.74A	24.68±1.26A

[a] Color parameters are lightness (*L**), redness (*a**), and yellowness (*b**).

[b] Means ± standard deviations from three replications. Within the same storage time and produce type, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

Table III-6. Maximum force (N) required for breakage of spinach leaves and tomatoes treated in combination with UVC and ClO_2 gas (10 ppmv) during storage for 7 days.

Day	Maximum force (N)			
	Spinach leaves		Tomatoes	
	Control	UVC + ClO_2 gas	Control	UVC + ClO_2 gas
0	47.23±0.93A[a]	47.82±0.84A	10.68±0.84A	10.21±0.77A
2	47.25±0.42A	48.00±2.69A	10.92±1.04A	10.99±1.05A
4	47.18±2.78A	46.67±1.52A	10.21±1.18A	10.76±2.37A
7	46.89±3.12A	45.14±1.86A	9.28±0.22A	9.91±1.58A

[a] Means ± standard deviations from three replications. Within the same storage time and produce type, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

III-1.4. Discussion

Antimicrobial effects of ClO_2 gas on produce, including spinach and tomatoes, have been reported. *Salmonella* and *E. coli* O157:H7 on spinach leaves treated with 2.1 mg/l ClO_2 gas (generated by a sachet) for 1 h were reduced by 0.6 and 0.7 log CFU/g, respectively (Neal et al., 2013). Bhagat et al. (2010) reported that treatment with 0.5 mg/l ClO_2 gas for 12 min showed more than a 5 log reduction in *Salmonella* and *L. monocytogenes* on tomato skin surfaces. Treatment with 8 mg/l ClO_2 gas for 60s and 10 mg/l ClO_2 gas for 180 s reduced levels of *Salmonella* on Roma tomatoes by 2.94 and 4.87 log CFU/cm^2, respectively (Trinetta et al., 2013). However, the concentration of ClO_2 gas used in previous studies was excessive (about 180-3600 ppmv). These concentrations were much higher than a LC50 value (32 ppmv, 90 mg/m^3) determined for rats as a single exposure (Dobson, 2002).

The antimicrobial effects of UVC on produce decontamination have also been evaluated by several studies. Sommers et al. (2010) reported a UVC dose of 5 kJ/m^2 inactivated 3.02 and 2.59 log of *Salmonella* spp. and *L. monocytogenes* on the surface of Roma tomatoes, respectively. A UVC dose of 4.9 kJ/m^2 achieved 1.79, 2.59, and 1.80 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on lettuce, respectively (Sommers et al., 2010). However, the poor penetration of UV light into solids is a significant limitation of UV light for decontamination of foods (Murdoch et al., 2010). Also, Mukhopadhyay et al. (2014)

reported that produce surface characteristics such as surface roughness and the location of foodborne pathogens on produce greatly influence the efficacy of UVC treatment.

In the present study, UVC was applied as hurdle technology to reduce the concentration of ClO_2 gas. As treatment time increased, the combination of UVC and ClO_2 gas (5 ppmv) caused similar microbial reductions of the three foodborne pathogens when compared with 10 ppmv ClO_2 gas treatments alone. In case of *E. coli* O157:H7, combined treatment with UVC and ClO_2 gas (5 ppmv) was more effective than 10 ppmv ClO_2 gas alone after 20 min of exposure. Park and Kang (2015) revealed that treatment with 30 ppmv of ClO_2 gas for 20 min at 90% RH caused 5.78, 5.68, and 4.86 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves, respectively. In this study, a combination treatment of UVC and ClO_2 gas (10 ppmv) for 20 min resulted in 5.17, 5.47, and 4.32 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves, respectively. Also, exposure to 20 ppmv of ClO_2 gas for 15 min at 90% RH caused more than 5.92 and 5.67 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes, respectively (unpublished). In the present study, the combination treatment of UVC and ClO_2 gas (10 ppmv) for 15 min resulted in 5.62 and 5.46 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. These results suggest that combined treatment with UVC and ClO_2 gas could reduce the concentration of ClO_2 gas, while ensuring microbial safety.

Combination treatments of UVC with chemical sanitizers have been widely used for inactivating microorganisms in food products (Jiang et al., 2010). Hadjok et al. (2008) reported that a combined treatment of UVC (37.8 mJ/cm^2) and H_2O_2 (1.5% at 50 ℃) caused a 4.12 log reduction of *Salmonella* on iceberg lettuce, which was significantly higher compared to UVC or H_2O_2 treatment alone. Combined treatment with UVC and O_3 achieved a maximum total microbial reduction of 6.6 log CFU/ml after 60 min treatment, whereas 4.0 and 5.9 log CFU/ml reductions, respectively, were achieved by UVC and O_3 treatment alone (Selma et al., 2008). The synergistic effect was observed in inactivating *Cronobacter sakazakii*, *Staphylococcus aureus*, *S.* Typhimurium, and *E. coli* when sodium hypochlorite and UV radiation were treated in combination (Ha and Ha, 2009).

In the present study, most combinations of UVC and ClO_2 gas showed only additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves as treatment time increased. However, synergistic effects in inactivating *E. coli* O157:H7 and *S.* Typhimurium on tomatoes were observed after combined treatment of UVC and ClO_2 gas (10 ppmv) for 15 min or more. In the case of *L. monocytogenes*, the existence of a synergistic effect was deduced after 20 min treatment. This difference may due to different surface characteristics of spinach leaves and tomatoes. Foodborne pathogens on tomatoes might be more easily exposed to UVC and ClO_2 gas than spinach leaves as tomatoes have smoother surfaces than spinach leaves.

The underlying inactivation mechanisms of the combined treatment of UVC and ClO_2 gas are not well understood. UVC radiation inactivates microorganisms by damaging their nucleic acid, thereby blocking cell replication (Koutchma, 2009). It has also been suggested that photons could interact with cell envelope components and favor the oxidation of unsaturated fatty acid residues of lipids and phospholipids (Koutchma et al., 2004, Montgomery, 1985). The mechanism of inactivation by ClO_2 has been postulated by several studies. Antimicrobial effects of ClO_2 are primarily due to oxidative attack on cell surface membrane proteins, including proteins involved in transport (Jeng and Woodworth, 1990). It is related to the denaturation of constituent proteins for cellular integrity and function, and the loss of permeability control of the outer membrane (Berg et al., 1986).

To clarify the mechanism of the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas, membrane damage to bacterial cells caused by UVC, ClO_2 gas, and UVC-ClO_2 gas simultaneous treatment was evaluated. Since cytoplasmic substances may have extracted out to the suspension via the permeabilized cell wall and membrane, we investigated whether leakage of UV-absorbing materials from cytoplasm had occurred after each treatment by using a spectrophotometer. Increasing treatment time of ClO_2 gas (10 ppmv) led to greater leakage of intracellular compounds for all three organisms tested in this study, whereas UVC treatment does not increase leakage of cytoplasmic materials. The synergism of UVC-ClO_2 gas simultaneous treatment was observed through

spectrophotometric measurements of leakage of UV-absorbing materials (260 nm) of *E. coli* O157:H7 and *S.* Typhimurium. Significant ($p < 0.05$) differences between the sum of levels of UV-absorbing substances of cells suspensions treated individually with UVC and ClO_2 gas (10 ppmv) and those achieved with combination treatment were observed after 15 min treatments. Quantitative results of cell membrane damage measured by UV-absorbing substances were consistent with TEM analysis (FIG. II-4). Also, the results show that there is a correlation between increased leakage of cytoplasmic materials and an increased level of inactivation determined by counts of CFU. These results provide evidence that damage to the cell membrane and changes to membrane permeability are involved in the synergistic lethal effect of the combination treatment of UVC and ClO_2 gas.

Cell membrane damage or permeability could affect the antimicrobial effect of ClO_2 gas, because for bactericides to be more effective, they must penetrate the cell envelope and attain a concentration high enough to exert their antimicrobial action (Virto et al., 2005). Also, the radical yielding UV photolysis of chlorine species could affect synergistic effect of the combination treatment of UVC and ClO_2 gas. Nowell and Hoigne (1992) reported that the UV/chlorine process produced ·OH radical, and the production of ·OH radicals has enabled the UV/chlorine process to become a potential advanced oxidation process (AOP). Sichel et al. (2011) reported the radical produced by UV/ClO_2 AOP could degrade the emerging contaminants.

In conclusion, as treatment time increased the combination treatment of UVC and ClO_2 gas could show additive or synergistic effects in the inactivation of three foodborne pathogens depending on type of produce and ClO_2 gas concentration. The results of this study indicate that the mechanism of the synergistic effect was related to membrane damage, followed by changes to membrane permeability. Food sample quality was maintained during 7 days of storage after combined treatment with all combinations of UVC and ClO_2 gas (10 ppmv). The results of this study suggest that the combination treatment of UVC and ClO_2 gas could be an alternative intervention for reducing the concentration of ClO_2 gas while ensuring microbial safety.

III-2. Combination treatment of ClO_2 gas and aerosolized sanitizer for inactivating foodborne pathogens on produce

III-2.1. Introduction

Consumption of fresh produce has increased because of its health benefits (Perni et al., 2008). However, several foodborne outbreaks related to the presence of pathogenic bacteria in fresh produce have been reported in recent years (Fernández and Thompson, 2012). Fresh spinach and spinach-containing products were implicated in an outbreak of *Escherichia coli* O157:H7 (CDC, 2006; Maki, 2006) which infected a total of 205 persons and resulted in 4 deaths (Wendel et al., 2009). In 2012, a total of 33 persons infected with *E. coli* O157:H7 traced to organic spinach and spring mix blend was reported from 5 US states (CDC, 2012). Tomatoes were associated with more than 14 outbreaks of foodborne illness between 1996 and 2008, and accounted for 17% of all produce-associated outbreaks in the United States during that period (Gravani, 2009).

Chlorine dioxide (ClO_2) has emerged as a promising non-thermal sanitizing technology for fresh produce in recent years (Bhagat et al., 2010). Several factors such as gas concentration, relative humidity (RH), treatment time, and temperature could affect the antimicrobial effect of ClO_2 gas. Especially, the combination of gas concentration and RH shows a synergistic effect (Han et al, 2001a; Park and Kang, 2015). The antimicrobial effect of ClO_2 gas has been evaluated on fresh produce such as spinach (Neal et al., 2012), potatoes (Wu and Rioux, 2010), mung bean sprouts (Prodduk et al., 2014), lettuce (Mahmoud and Linton, 2008), onions,

cabbage (Sy et al., 2005), cantaloupe (Mahmoud et al., 2008), and strawberries (Han et al., 2004). However, the concentration of ClO_2 gas used in previous studies was excessive (Morino et al., 2011).

Combinations of different technologies, known as hurdle technology, could be an alternative to the use of high ClO_2 gas concentrations. Combined treatments could achieve required levels of food safety and the maintenance of organoleptic qualities of foods, while decreasing the intensity of each hurdle, that is, the antimicrobial concentration (Leistner and Gorris, 1995). Studies which evaluated sanitizer-sanitizer or sanitizer-novel technique combinations have both drawn great attention (Huang and Chen, 2011; Singh et al., 2002).

Aerosolization, another non-thermal technology, is the dispersion of a liquid material as a fine mist in air (Oh et al., 2005). Some studies have investigated the efficacy of aerosolized sanitizers for inhibiting foodborne pathogens on fresh produce. Aerosolized peroxyacetic acid, hydrogen peroxide, and malic acid were effective for controlling *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on lettuce and spinach leaves (Choi et al., 2012; Huang et al., 2012; Oh et al., 2005). Not only the antimicrobial effect, but also the ability to control humidity is advantage of aerosolized sanitizers for combination with ClO_2 gas. Since aerosolized sanitizer exists as a fine mist dispersed in air, it can be used to control RH of the ClO_2 gas treatment chamber. Thus, aerosolized sanitizers in combination

with ClO_2 gas could enhance the inactivation efficacy of ClO_2 gas by maintaining conditions of high RH.

The objective of this study was to evaluate the antimicrobial effect of ClO_2 gas and aerosolized sanitizer, when applied alone or in combination, on the survival of inoculated *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves and tomato surfaces. Also, any changes in color and texture of samples were assessed.

III-2.2. Materials and Methods

Bacterial strains and cell suspension. Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were provided by the bacterial culture collection of the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea), for this study. All strains of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were cultured individually in 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 ℃ for 24 h, followed by centrifugation (4000 × g for 20 min at 4 ℃) and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile BPW, corresponding to ca. 10^7-10^8 CFU/ml. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* strains were combined to make culture cocktails for use in experiments.

Sample preparation and inoculation. Spinach and whole tomatoes were purchased from a local market (Seoul, South Korea). Spinach leaves were trimmed to approximately 5 cm × 3 cm in size, and the outer surface of tomatoes was cut into 5 cm × 2 cm size pieces. Prepared spinach leaves and tomato surface samples were placed on aluminum foil in a laminar flow biosafety hood, and 0.1 ml of culture cocktail was inoculated onto one side of each prepared sample by depositing

droplets with a micropipettor at 15-20 locations. Samples were dried in the hood for 1 h at 22 ± 2 ℃ with the fan running.

Combined treatment of ClO_2 gas and aerosolized sanitizer. The combined treatment of ClO_2 gas and aerosolized sanitizer was conducted in the treatment apparatus described previously (Park and Kang, 2015). Peracetic acid (PAA) (Omega Chemical, Gyeongbuk, Korea) was used as an aqueous sanitizer and diluted with distilled water to a concentration of 80 ppm. The U.S. Food and Drug Administration (FDA) approved the use of PAA for sanitizing fruits and vegetables at concentrations that do not exceed 80 ppm in wash water (Anonymous, 2000). Inoculated spinach leaves and tomatoes were placed in the treatment chamber and covered with a plastic lid. For treatments with ClO_2 gas alone, samples were subjected to 5 or 10 ppmv ClO_2 gas for 20 min. The RH of the treatment chamber was adjusted with distilled water to 90% with an accuracy of ± 2%. For treatment with only aerosolized PAA, samples were exposed to 80 ppm of aerosolized PAA for 20 min. During treatment, the RH of the treatment chamber was adjusted with aerosolized PAA to 90 ± 2%. For combined treatments, samples were subjected to ClO_2 gas (5 or 10 ppmv) and 80 ppm of aerosolized PAA for 20 min. The RH of the treatment chamber was adjusted with aerosolized PAA to 90 ± 2% during treatment. All experiments were performed at 22 ± 2 ℃. When the desired ClO_2 gas concentration and RH were achieved, the plastic lid was removed and the inoculated side of spinach leaves and tomatoes were exposed to ClO_2 gas. Samples were

withdrawn after 5, 10, 15, and 20 min exposure to each treatment, and treated samples were used to determine surviving bacterial populations. These experiments were repeated three times.

Bacterial enumeration. Treated and untreated (control) spinach leaves (10 ± 0.2 g) and one piece of tomato were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 or 30 ml of neutralizing buffer (Difco), respectively. Stomacher bags were homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, 1 ml sample aliquots were tenfold serially diluted in 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto selective media. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Modified Oxford Medium (MOX; Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Where low numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates to lower the detection limit. The plates were incubated at 37 ℃ for 24–48 h, and colonies were counted after incubation.

For the resuscitation of injured *E. coli* O157:H7, phenol red agar base (Difco) with 1% sorbitol (SPRAB) was used (Rhee et al., 2003). One hundred microliter of sample or diluent was spread-plated onto SPRAB and incubated at 37 ℃ for 24 h. Injured cells of *S.* typhimurium and *L. monocytogenes* were enumerated using the overlay (OV) method proposed by Kang and Fung (1999, 2000). One hundred

microliter of sample or diluent was spread-plated onto TSA and incubated at 37 ℃ for 2 h to allow injured cells to resuscitate before overlaying with 7 mL of XLD (OV-XLD) or MOX (OV-MOX) for *S.* Typhimurium and *L. monocytogenes*, respectively. The plates were incubated at 37 ℃ for 22 h after the overlay solidified. Where low numbers of surviving cells were anticipated, 250 µl of undiluted cell suspension was plated onto four plates of each respective medium.

Measurement of color and texture of samples. Treated spinach leaves and tomatoes (uninoculated) were stored at 4 and 12 ℃ for 7 days, respectively, to identify quality changes during storage following each treatment. Color values (Hunter's L, a, b) of spinach leaves and tomatoes were measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each sample. The texture of spinach leaves and tomatoes was evaluated with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set and cylinder probe with a 4 mm diameter, respectively. Twenty grams of spinach leaves was placed onto the press holder with the stems positioned perpendicular to the path of the blade, and a blade was moved down at 2 mm/s (path length 10 mm). For tomatoes, the loading rate and path length were set at 2 mm/s and 10 mm. Three measurements were performed with independently-prepared samples for each treatment. Texturepro CT software (Brookfield Engineering Laboratories, Inc.) was used to record maximum force.

Statistical analysis. All experiments were done in triplicate. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

III-2.3. Results

Effects of ClO_2 gas, aerosolized sanitizer, and combination treatment on populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. Microbial reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves by ClO_2 gas, aerosolized PAA, and combined treatment of both technologies simultaneously are shown in Table III-7 and 8. In general, antimicrobial effects of combined treatments of ClO_2 gas (5 ppmv) and aerosolized PAA (80 ppm) were not superior to those of individual treatments during 10 min exposure. After 15 min treatment, the combination of both treatments resulted in more significant ($p < 0.05$) microbial reduction of the three foodborne pathogens than each treatment alone. Levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells following 15 min treatment with ClO_2 gas were reduced by 1.95, 1.76, and 1.26 log, respectively. Exposure to aerosolized PAA for 15 min resulted in 1.58, 1.71, and 0.79 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (5 ppmv) and aerosolized PAA for 15 min resulted in 2.64, 2.32, and 2.31 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (5 ppmv) and aerosolized PAA showed an additive effect after 20 min treatment: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from the sum of individual treatments. Exposure to

5 ppmv of ClO_2 gas for 20 min resulted in 2.33, 2.17, and 1.97 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Treatment with aerosolized PAA for 20 min caused 2.25, 1.85, and 0.82 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (5 ppmv) and aerosolized PAA for 20 min resulted in 4.21, 3.90, and 2.64 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Similarly, most combinations of ClO_2 gas (10 ppmv) and aerosolized PAA showed additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after 15 min treatment. Exposure to 10 ppmv of ClO_2 gas for 20 min resulted in 3.39, 3.29, and 3.36 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA for 20 min produced 5.36, 5.06, and 4.06 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

Table III-9 and 10 shows microbial reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes following ClO_2 gas, aerosolized PAA, and combined treatment of both technologies simultaneously. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes showed similar reduction patterns to those on spinach leaves. As treatment time increased, most combinations of ClO_2 gas and aerosolized PAA showed additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, with inactivation generally

superior to that of each treatment applied individually. Levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells following 20 min treatment of ClO_2 gas (10 ppmv) were reduced by 3.85, 3.54, and 3.40 log, respectively. Treatment with aerosolized PAA for 20 min caused 1.33, 1.27, and 0.81 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA for 20 min resulted in 5.09, 5.24, and 4.51 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

Combined treatment of ClO_2 gas and aerosolized PAA produced injured cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves (Table III-11 and 12). In case of tomatoes, combined treatment of ClO_2 gas and aerosolized PAA generally did not produce injured cells of three pathogens on tomatoes (Table III-13 and 14).

Table III-7. Log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with aerosolized PAA (80 ppm), 5 ppmv ClO_2 gas, and both technologies simultaneously (PAA-ClO_2 gas)[a].

Bacteria	Treatments	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	Aerosolized PAA	0.26±0.22Aa[b]	0.59±0.26Aa	1.47±0.16Ba	1.58±0.23Ba	2.25±0.10Ca
	ClO_2 gas	0.67±0.24Ab	1.34±0.24Bb	1.86±0.40BCa	1.95±0.23BCa	2.33±0.54Ca
	PAA + ClO_2 gas	0.56±0.10Aab	0.95±0.11Aab	1.81±0.25Ba	2.64±0.49Cb	4.21±0.47Db
S. Typhimurium	Aerosolized PAA	0.68±0.18Aa	1.10±0.09Ba	1.58±0.12Ca	1.71±0.37Ca	1.85±0.21Ca
	ClO_2 gas	0.55±0.16Aa	1.09±0.22Ba	1.57±0.14Ca	1.76±0.30Ca	2.17±0.26Da
	PAA + ClO_2 gas	0.71±0.09Aa	1.23±0.28Ba	1.71±0.17Ca	2.32±0.06Db	3.90±0.43Eb
L. monocytogenes	Aerosolized PAA	0.10±0.21Aa	0.59±0.20Ba	0.76±0.20Ba	0.79±0.20Ba	0.82±0.30Ba
	ClO_2 gas	0.38±0.24Aa	0.74±0.35ABa	0.82±0.35ABa	1.26±0.33Ba	1.97±0.40Cb
	PAA + ClO_2 gas	0.33±0.33Aa	0.83±0.27ABa	1.21±0.36Ba	2.31±0.55Cb	2.64±0.28Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-8. Log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves treated with aerosolized PAA (80 ppm), 10 ppmv ClO_2 gas, and both technologies simultaneously (PAA-ClO_2 gas)[a].

Bacteria	Treatments	Log reduction (log CFU/g)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	Aerosolized PAA	0.26±0.22Aa[b]	0.61±0.29Aa	1.49±0.18Ba	1.61±0.22Ba	2.27±0.12Ca
	ClO_2 gas	0.85±0.25Ab	1.12±0.36Aab	1.96±0.11Bab	2.48±0.37Cb	3.39±0.01Db
	PAA + ClO_2 gas	0.91±0.15Ab	1.63±0.09Bb	2.47±0.42Bc	3.92±0.31Dc	5.36±0.30Ec
S. Typhimurium	Aerosolized PAA	0.68±0.18Aa	1.03±0.24Aab	1.62±0.18Ba	1.75±0.27Ba	1.89±0.10Ba
	ClO_2 gas	0.39±0.51Aa	0.68±0.23Aa	1.58±0.16Ba	2.69±0.19Cb	3.29±0.17Db
	PAA + ClO_2 gas	0.91±0.11Aa	1.39±0.30Bb	2.07±0.03Cb	3.58±0.42Dc	5.06±0.32Ec
L. monocytogenes	Aerosolized PAA	0.12±0.04Aa	0.61±0.22Ba	0.78±0.19Ba	0.81±0.23Ba	0.84±0.34Ba
	ClO_2 gas	0.41±0.02Aab	0.97±0.19Bab	1.39±0.08Bb	2.52±0.53Cb	3.36±0.32Db
	PAA + ClO_2 gas	0.68±0.29Ab	1.36±0.37Bb	1.89±0.39Bb	3.46±0.23Cc	4.06±0.22Dc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-9. Log reductions of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* on tomatoes treated with aerosolized PAA (80 ppm), 5 ppmv ClO_2 gas, and both technologies simultaneously (PAA-ClO_2 gas)[a].

Bacteria	Treatments	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	Aerosolized PAA	0.24±0.11Aa[b]	0.49±0.13Aa	0.88±0.28Ba	0.95±0.17BCa	1.23±0.08Ca
	ClO_2 gas	0.69±0.48Aa	1.27±0.33Bb	1.61±0.18BCb	1.92±0.17CDb	2.34±0.18Db
	PAA + ClO_2 gas	0.26±0.21Aa	1.23±0.29Bb	2.00±0.16Bc	2.65±0.28Dc	3.83±0.14Ec
S. Typhimurium	Aerosolized PAA	0.20±0.13Aa	0.40±0.28Aa	0.53±0.09ABa	1.07±0.56BCa	1.18±0.30Ca
	ClO_2 gas	0.50±0.09Ab	0.96±0.15ABb	1.20±0.22BCb	1.62±0.50Ca	2.24±0.27Db
	PAA + ClO_2 gas	0.50±0.16Ab	1.14±0.33Bb	1.94±0.26Cc	2.72±0.24Db	3.74±0.40Ec
L. monocytogenes	Aerosolized PAA	0.42±0.09Aa	0.72±0.21ABa	0.73±0.37ABa	0.91±0.09Ba	0.86±0.17Ba
	ClO_2 gas	0.55±0.08Aa	0.86±0.13ABa	1.04±0.35BCa	1.36±0.11CDb	1.57±0.15Db
	PAA + ClO_2 gas	0.56±0.07Aa	0.94±0.46ABa	1.33±0.08BCa	1.59±0.06CDc	1.94±0.05Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-10. Log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes treated with aerosolized PAA (80 ppm), 10 ppmv ClO_2 gas, and both technologies simultaneously (PAA-ClO_2 gas)[a].

Bacteria	Treatments	Log reduction (log CFU/cm^2)				
		1 min	5 min	10 min	15 min	20 min
E. coli O157:H7	Aerosolized PAA	0.34±0.05Aa[b]	0.60±0.23Aa	0.98±0.23Ba	1.05±0.13BCa	1.33±0.18Ca
	ClO_2 gas	0.97±0.05Ab	1.22±0.58Aa	2.46±0.32Bb	3.16±0.06Cb	3.85±0.38Db
	PAA + ClO_2 gas	0.40±0.20Aa	0.99±0.40Aa	2.62±0.46Bb	3.65±0.39Cc	5.09±0.35Dc
S. Typhimurium	Aerosolized PAA	0.30±0.13Aa	0.49±0.28Aa	0.62±0.12Aa	1.16±0.45Ba	1.27±0.18Ba
	ClO_2 gas	0.89±0.34Ab	1.33±0.20ABb	1.89±0.32Bb	2.57±0.55Cb	3.54±0.31Db
	PAA + ClO_2 gas	0.66±0.17Aab	1.23±0.44Bb	1.91±0.31Cb	3.81±0.13Dc	5.24±0.25Ec
L. monocytogenes	Aerosolized PAA	0.63±0.13Aa	0.67±0.16Aa	0.68±0.33Aa	0.86±0.04Aa	0.81±0.13Aa
	ClO_2 gas	0.61±0.28Aa	1.03±0.13ABa	1.43±0.08Bb	2.28±0.49Cb	3.40±0.25Db
	PAA + ClO_2 gas	0.65±0.14Aa	0.74±0.15Aa	1.48±0.06Bb	3.01±0.31Cc	4.51±0.20Dc

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.
[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). Means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-11. Levels of surviving and injured cells on spinach leaves following combined treatment of ClO_2 gas (5 ppmv) and aerosolized PAA.

Treatment time	Log reduction (log CFU/g)					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
	PAA-ClO_2 gas		PAA-ClO_2 gas		PAA-ClO_2 gas	
	SPRAB[a]	SMAC	OV-XLD	XLD	OV-MOX	MOX
5 min	0.99±0.45A[b]	0.95±0.11A	0.82±0.12A	1.23±0.28A	0.34±0.11A	0.83±0.27B
10 min	1.65±0.20A	1.81±0.25A	1.34±0.11A	1.71±0.17B	0.76±0.10A	1.21±0.36A
15 min	1.92±0.27A	2.64±0.49A	1.76±0.21A	2.32±0.06B	1.25±0.22A	2.31±0.55B
20 min	3.20±0.25A	4.21±0.47B	2.97±0.13A	3.90±0.43B	2.06±0.12A	2.64±0.28B

[a] SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate agar; OV-XLD, overlay XLD agar on TSA; MOX, modified Oxford agar; OV-MOX, overlay MOX agar on TSA.
[b] Means with different uppercase letters in the same row are significantly different ($p < 0.05$).

Table III-12. Levels of surviving and injured cells on spinach leaves following combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA.

Treatment time	Log reduction (log CFU/g)					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
	PAA-ClO_2 gas		PAA-ClO_2 gas		PAA-ClO_2 gas	
	SPRAB[a]	SMAC	OV-XLD	XLD	OV-MOX	MOX
5 min	0.91±0.36A[b]	1.63±0.09B	0.91±0.06A	1.39±0.30A	0.48±0.16A	1.36±0.37B
10 min	2.15±0.37A	2.47±0.42A	1.32±0.06A	2.07±0.03B	1.14±0.20A	1.89±0.39B
15 min	3.08±0.61A	3.92±0.31A	2.50±0.33A	3.58±0.42B	1.96±0.55A	3.46±0.23B
20 min	5.02±0.11A	5.36±0.30A	4.79±0.11A	5.06±0.32A	3.98±0.05A	4.06±0.22A

[a] SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate agar; OV-XLD, overlay XLD agar on TSA; MOX, modified Oxford agar; OV-MOX, overlay MOX agar on TSA.
[b] Means with different uppercase letters in the same row are significantly different ($p < 0.05$).

Table III-13. Levels of surviving and injured cells on tomatoes following combined treatment of ClO_2 gas (5 ppmv) and aerosolized PAA.

Treatment time	Log reduction (log CFU/cm^2)					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
	PAA-ClO_2 gas		PAA-ClO_2 gas		PAA-ClO_2 gas	
	SPRAB[a]	SMAC	OV-XLD	XLD	OV-MOX	MOX
5 min	1.00±0.40A[b]	1.23±0.29A	0.87±0.26A	1.14±0.33A	0.37±0.07A	0.94±0.46A
10 min	1.27±0.06A	2.00±0.16B	1.33±0.39A	1.94±0.26A	0.68±0.08A	1.33±0.08B
15 min	2.45±0.11A	2.65±0.28A	2.40±0.11A	2.72±0.24A	1.43±0.09A	1.59±0.06A
20 min	3.47±0.37A	3.83±0.14A	3.52±0.50A	3.74±0.40A	1.93±0.55A	1.94±0.05A

[a] SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate agar; OV-XLD, overlay XLD agar on TSA; MOX, modified Oxford agar; OV-MOX, overlay MOX agar on TSA.
[b] Means with different uppercase letters in the same row are significantly different ($p < 0.05$).

Table III-14. Levels of surviving and injured cells on tomatoes following combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA.

Treatment time	Log reduction (log CFU/cm^2)					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
	PAA-ClO_2 gas		PAA-ClO_2 gas		PAA-ClO_2 gas	
	SPRAB[a]	SMAC	OV-XLD	XLD	OV-MOX	MOX
5 min	0.96±0.10A[b]	0.99±0.40A	0.96±0.49A	1.23±0.44A	0.35±0.16A	0.74±0.15B
10 min	2.26±0.12A	2.62±0.46A	1.71±0.26A	1.91±0.31A	1.44±0.08A	1.48±0.06A
15 min	3.49±0.08A	3.65±0.39A	3.47±0.20A	3.81±0.13A	2.59±0.12A	3.01±0.31A
20 min	4.92±0.53A	5.09±0.35A	5.21±0.41A	5.24±0.25A	4.47±0.10A	4.51±0.20A

[a] SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate agar; OV-XLD, overlay XLD agar on TSA; MOX, modified Oxford agar; OV-MOX, overlay MOX agar on TSA.
[b] Means with different uppercase letters in the same row are significantly different ($p < 0.05$).

The quality changes of spinach leaves and tomatoes during storage. During storage, the *a** value of spinach leaves decreased while the *b** value increased. However, there were no significant ($p > 0.05$) differences in Hunter's color values (*L**, *a**, *b**) between untreated samples (control) and those treated with combined ClO_2 gas (10 ppmv) and aerosolized PAA during 7 days storage (Table III-15). In the case of tomatoes, the *L** and *a** values increased during storage. However, there were also no significant ($p > 0.05$) differences in Hunter's color values (*L**, *a**, *b**) between controls and those treated with combined ClO_2 gas (10 ppmv) and aerosolized PAA during storage.

Table III-16 shows the effects of combined treatment of ClO_2 gas (10 ppmv) and aerosolized PAA on the texture of samples. There were no significant ($p > 0.05$) differences in texture between control and treated samples during storage for 7 days.

Table III-15. Changes in color values[a] of spinach leaves and tomatoes combination-treated with ClO_2 gas (10 ppmv) and aerosolized PAA (80 ppm) during storage for 7 days.

Day	Treatment			
	Spinach leaves		Tomatoes	
	Control	ClO_2 + aerosolized PAA	Control	ClO_2 + aerosolized PAA
		*L**		
0	40.51±1.27A[b]	39.93±1.31A	44.07±0.42A	43.15±0.58A
2	40.68±1.12A	40.50±1.54A	44.55±1.03A	44.29±0.55A
4	40.51±1.02A	40.21±1.04A	42.52±0.64A	41.41±1.25A
7	39.47±1.35A	38.85±0.64A	41.03±1.02A	41.78±0.65A
		*a**		
0	-6.91±0.57A	-6.99±0.70A	18.37±2.10A	19.63±0.68A
2	-6.93±0.83A	-7.74±0.47A	20.27±2.19A	18.83±0.35A
4	-7.84±0.66A	-8.40±0.57A	19.38±1.13A	19.35±1.01A
7	-8.86±0.76A	-9.02±0.62A	20.33±0.93A	20.02±0.29A
		*b**		
0	9.14±0.60A	9.46±0.97A	19.43±1.93A	22.23±1.42A
2	8.97±1.19A	10.01±1.00A	24.50±0.57A	25.37±0.61A
4	10.32±1.25A	10.54±0.98A	25.26±2.23A	24.30±0.77A
7	12.76±1.64A	12.53±0.63A	24.22±0.58A	24.17±0.20A

[a] Color parameters are lightness (*L**), redness (*a**), and yellowness (*b**).

[b] Means ± standard deviations from three replications. Within the same storage time and type of vegetable, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

Table III-16. Maximum force (N) required for breakage of spinach leaves and tomatoes combination-treated with ClO_2 gas (10 ppmv) and aerosolized PAA (80 ppm) during storage for 7 days.

Day	Maximum force (N)			
	Spinach leaves		Tomatoes	
	Control	ClO_2 + aerosolized PAA	Control	ClO_2 + aerosolized PAA
0	48.16±1.87A[a]	47.12±2.71A	10.85±0.73A	10.75±0.54A
2	49.16±2.33A	49.27±2.10A	10.77±0.79A	10.34±0.66A
4	47.12±2.78A	48.22±2.56A	9.90±1.01A	10.53±0.74A
7	46.85±3.16A	44.34±2.26A	9.12±0.06A	9.90±0.91A

[a] Means ± standard deviations from three replications. Within the same storage time and type of vegetable, means with the same uppercase letters within a row are not significantly different ($p > 0.05$).

III-2.4. Discussion

Several studies have reported antimicrobial effects of ClO_2 gas on produce including spinach and tomatoes. Neal et al. (2012) reported that *Salmonella* and *E. coli* O157:H7 on spinach leaves exposed to 2.1 mg/l ClO_2 gas (generated by a sachet) for 1 h were reduced by 0.6 and 0.7 log CFU/g, respectively. Treatment with 10 mg/l ClO_2 gas for 180 s reduced levels of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* on Roma tomatoes (*Lycopersicon esculentum*) by 4.8, 3.6, and 3.0 log CFU/cm^2, respectively (Trinetta et al., 2013). Bhagat et al. (2010) reported that more than a 5 log reduction in *Salmonella* and *L. monocytogenes* was observed on tomato skin surfaces after treatment with 0.5 mg/l ClO_2 gas for 12 min. However, the concentration of ClO_2 gas used in previous reports was excessive (about 180-3600 ppmv). These concentrations were much higher than a LC50 value (32 ppmv, 90 mg/m^3) determined for rats as a single exposure (Dobson, 2002).

In the present study, aerosolized PAA was applied as hurdle technology to reduce the concentration of ClO_2 gas. Some studies have evaluated the antimicrobial effect of aerosolized sanitizers on produce decontamination. Oh et al. (2005) reported that aerosolized peroxyacetic acid resulted in a 3-4 log reduction in populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on lettuce leaves. *E. coli* O157:H7 on spinach and lettuce leaves exposed to 2% malic acid for 30 min were reduced by 2.1 and 2.5 log CFU/g, respectively. Huang et al. (2012)

reported that treatments of aerosolized sanitizers (2.5% lactic acid + 2% allyl isothiocyanate) resulted in > 4.8 log reduction of *E. coli* O157:H7 on spinach leaves. Treatment of inoculated lettuce with aerosolized malic acid (2%) for 30 min caused 2.6 and 2.5 log reductions of *S.* Typhimurium and *E. coli* O157:H7, respectively (Choi et al., 2012).

The combination of ClO_2 gas (5 ppmv) and aerosolized PAA (80ppm) was more effective than 10 ppmv ClO_2 gas alone for inactivating the three foodborne pathogens, except for *L. monocytogenes*, on spinach leaves and tomato surfaces after 20 min treatment. Our study (Park and Kang, 2015) revealed that exposure to 30 ppmv of ClO_2 gas for 20 min at 90% RH resulted in 5.8, 5.7, and 4.9 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves, respectively. In this study, a combination treatment of ClO_2 gas (10 ppmv) and aerosolized PAA (80 ppm) for 20 min yielded 5.4, 5.1, and 4.1 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves, respectively. Also, treatment with 20 ppmv of ClO_2 gas for 15 min at 90% RH resulted in greater than 5.9, 5.7, and 5.1 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on tomatoes, respectively (unpublished). In this study, the combination treatment of ClO_2 gas (10 ppmv) and aerosolized PAA for 20 min resulted in 5.1, 5.2, and 4.5 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. These results suggest that the

combination treatment of ClO_2 gas and aerosolized PAA could reduce the concentration of ClO_2 gas required, while still ensuring microbial safety.

The combination of gas concentration and RH represents a synergistic effect (Han et al, 2001; Park and Kang, 2015). Reductions of *E. coli* O157:H7 on green peppers increased from 1.9 to 4.0 log CFU/5g as RH increased from 55 to 95% when green peppers were treated with 0.3 mg/l ClO_2 gas at 15 ℃ (Han et al., 2001). Several studies have also evaluated the antimicrobial effect of ClO_2 gas under conditions of high RH (>80%) (Bhagat et al., 2011; Gómez-López et al., 2008; Popa et al., 2007; Vandekinderen et al., 2009). Its own antimicrobial effect as well as the ability to control RH is an advantage of utilizing aerosolized sanitizers in combination with ClO_2 gas. Combination treatment could easily be constructed by substituting an aqueous sanitizer for distilled water in an ultrasonic nebulizer without adding any additional treatment step. Also, combined treatment of ClO_2 gas and aerosolized sanitizers may be more effective in reducing foodborne pathogens internalized or present in low numbers in inaccessible areas of produce, because both technologies have better penetration properties than aqueous sanitizers (Han et al., 2001b; Hiom et al., 2003).

In the present study, most combinations of ClO_2 gas and aerosolized PAA showed additive effects in the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. It has been reported that the application of technologies in hurdles with complementary modes of action may have an impact on microbial

inactivation (Gallo et al., 2007; Sobrino-López and Martín-Belloso, 2008). However, ClO_2 gas and PAA have a similar mode of action, as they both are strong oxidizing agents, and disinfects by oxidizing of the cell membrane of vegetative bacterial cells (Benarde et al., 1965; Kitis, 2004). This might explain results of this study which demonstrate that the combination treatment of ClO_2 gas and aerosolized PAA represents only an additive effect on inactivation.

As injured cells of foodborne pathogens might be repaired under suitable conditions, it is a very important aspect that needs to be taken into account regarding food safety (García et al., 2005). In the present study, the occurrence of sublethally injured pathogens was assessed after combined treatment of ClO_2 gas and aerosolized PAA. Combined treatment of ClO_2 gas and aerosolized PAA produced more injured cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on spinach leaves than those on tomatoes. This difference may due to different surface characteristics of spinach leaves and tomatoes. Foodborne pathogens on tomatoes might be more easily exposed to ClO_2 gas and aerosolized PAA than spinach leaves as tomatoes have smoother surfaces than spinach leaves.

In conclusion, this study showed that the combination treatment of ClO_2 gas and aerosolized PAA showed additive effects in the inactivation of three foodborne pathogens with inactivation generally superior to that of each treatment applied individually, as treatment time increased. The color and texture of samples were maintained during 7 days of storage after combined treatment with ClO_2 gas (10

ppmv) and aerosolized PAA. The results of this study suggest that the combination treatment of ClO_2 gas and aerosolized PAA could be an alternative technology to reduce the concentration of ClO_2 gas while ensuring microbial safety.

III-3. Sequential treatment of ClO_2 gas and dry heat to inactivate foodborne pathogens on seeds

III-3.1. Introduction

The consumption of sprouted seed products has increased in recent decades because of their nutritional value (Nei et al., 2011; Xiao et al., 2014). However, consumption of sprouts has been associated with a number of foodborne disease outbreaks. Most outbreaks have been associated with alfalfa, mung bean, and radish seed sprouts contaminated with *Escherichia coli* O157:H7 and *Salmonella* spp. (Bang et al., 2011a; NACMCF, 1999). In the U. S., there were at least 33 outbreaks linked to sprouted seed products between 1998 and 2010 (Dechet et al., 2014). Sprouts from an organic farm were determined as a source of an *E. coli* O104:H4 outbreak in 2011, Germany, which caused about 4000 infections and 53 deaths (Uphoff et al., 2014). Also, sprouts-associated outbreaks in Japan (Watanabe et al., 1999), Canada (CFIA, 2005), Sweden, Finland, and Denmark (Emberland et al., 2007) have been reported.

Although the contamination levels of seeds are usually low in numbers (Jaquette et al., 1996; Taormina et al., 1999), the optimal temperature and humidity condition during the sprouting process are favorable for the rapid growth of pathogens (Feng et al., 2007; Stewart et al., 2001; Taormina and Beuchat, 1999). Therefore, assuring the absence of foodborne pathogens on seeds is regarded as a critical control point (Weiss and Hammes, 2005). The U.S. Food and Drug Administration (FDA)

recommends the application of 20,000 ppm of free chlorine from calcium hypochlorite during seed soaking (NACMCF, 1999). However, the effect of chlorine treatment has been reported to be limited (Holliday et al., 2001; Lang et al., 2000; Montville and Schaffner, 2004). Also, chlorine can combine with organic substances in water leading to the formation of harmful by-products such as trihalomethanes (THMs) (Dunnick and Melnick, 1993).

The application of dry heat treatments has been one of the most studied methods for the decontamination of seeds. Dry heat treatment could be easily applied in a seed sanitizing process. However, dry heat treatment alone requires 1 to 6 days to achieve more than 5 log reductions of *E. coli* O157:H7 and *Salmonella* (Feng et al., 2007; Hu et al., 2004; Neetoo and Chen, 2011). Combining two or more types of treatments, either simultaneously or in sequence, has been applied to achieve greater reductions in numbers of pathogens on seeds. Combined treatments of dry heat and other chemical or physical treatments have been studied including ethanol, oxalic acid, electrolyzed water (Bari et al., 2003), aqueous chlorine dioxide (ClO_2) (Bang et al., 2011b; Kim et al., 2010a), high hydrostatic pressure (Neetoo and Chen, 2011; Neetoo et al., 2009), and gamma irradiation (Bari et al., 2009), and each showed different levels of inactivation.

ClO_2 gas is a strong oxidizing agent with a broad antimicrobial spectrum and its antimicrobial efficacy is not greatly affected by pH or presence of organic matter (Beuchat, 1998; Trinetta et al., 2012). The objective of this study was to investigate

the efficacy of sequential treatments of ClO_2 gas and dry heat for eliminating *E. coli* O157:H7 and *Salmonella* Typhimurium on alfalfa and radish seeds, and evaluate the impact of this sequential treatment on seed viability. Also, the influence of surface morphology of seeds on the antimicrobial effect was examined by scanning electron microscopy analysis.

III-3.2. Materials and Methods

Bacterial strains and cell suspension. Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890) and *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104) obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea) were used in this study. All strains of *E. coli* O157:H7 and *S.* Typhimurium were inoculated individually into 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) and incubated for 24 h at 37 ℃. Overnight culture (1 ml) of each strain was spread onto three tryptic soy agar (TSA; Difco) plates to produce a bacterial lawn, which was followed by incubation for 24 h at 37 ℃. Ten ml of buffered peptone water (BPW; Difco) was added to each plate to harvest the bacterial lawn, and cell suspensions were made by rubbing the agar surface with a sterile swab (3M pipette swab, 3M Korea Ltd.) to dislodge cells. Cell suspensions (ca.10^{11}-10^{12} CFU/ml) were combined to construct a mixed culture cocktail, and augmented with BPW to yield 200 ml of culture cocktail in total with a final cell population of ca. 10^{9}-10^{10} CFU/ml.

Inoculation of seeds. Alfalfa and radish seeds were purchased from Danong (Gyeonggi-do, Korea) and stored at room temperature. Two hundred grams of alfalfa or radish seeds were added to 200 ml of mixed cell suspension and gently agitated for 10 min. The cell suspension was drained completely, and the seeds were

dried in a laminar flow hood for 18 h at 22 ± 1 ℃ before use in experiments. Moisture content (dry basis) of seeds was measured using a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH).

Sequential treatment with ClO_2 gas and dry heat. ClO_2 gas treatment was conducted in a treatment system described previously (Park and Kang, 2015). A tray (polypropylene, diameter × height, 180 × 75 mm) containing inoculated alfalfa or radish seeds (80 g) was fixed onto a vortex mixer (WiseMix VM-10; Daihan Wisd., Gangwon, South Korea) and placed in the treatment chamber. Seeds were treated with 150 ppmv ClO_2 gas at 22 ± 1 ℃ for 1 h. During treatment, seeds in the tray were continuously rotated by the vortex mixer and relative humidity (RH) of the treatment chamber was adjusted with distilled water to 90 ± 2%. Seeds used for dry heat treatment alone were treated the same way but without ClO_2 gas to adjust moisture content before dry heat treatment. Treated seeds were divided into 10 g portions and placed onto polystyrene trays (length × width × height, 89 × 89 × 25 mm). These trays were transferred to an oven (OV-11, JEIO Tech, Seoul, Korea Rep.) with the temperature set at 70 or 80 ℃. Alfalfa or radish seeds were heated for 0, 1, 3, or 5 h.

Enumeration of *E. coli* O157:H7 and *S.* Typhimurium. Each treated seed sample (10 g) was immediately transferred to a sterile filter stomacher bag (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 ml of neutralizing buffer (Difco), and homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes,

France). After homogenization, a 1 ml aliquot of sample was tenfold serially diluted in 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco) and Xylose Lysine Desoxycholate agar (XLD; Difco) were used as selective media for the enumeration of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Where low numbers of surviving cells were anticipated, 250 μl of undiluted sample was plated onto each of four plates. All plates were incubated at 37 ℃ for 24 h, and colonies were counted.

Phenol red agar base (Difco) with 1% sorbitol (SPRAB) was used for the resuscitation of injured *E. coli* O157:H7 (Rhee et al., 2003). One hundred microliter of sample or diluent was spread-plated onto SPRAB and incubated at 37 ℃ for 24 h. Injured cells of *S.* typhimurium were enumerated using the overlay (OV) method proposed by Kang and Fung (Kang and Fung, 1999; Kang and Fung, 2000). One hundred microliter of sample or diluent was spread-plated onto TSA and incubated at 37 ℃ for 2 h to allow injured cells to resuscitate before overlaying with 7 mL of XLD (OV-XLD). The plates were incubated at 37 ℃ for 22 h after the overlay solidified. Where low numbers of surviving cells were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium.

Determination of seed germination rate. Treated or untreated (control) seeds (n = 200) were placed on sterile cheesecloth in petri dishes (90 mm diameter), and periodically provided with distilled water to maintain the amount of moisture the seeds require for sprouting. The seeds were incubated at room temperature (22 ±

1 ℃) for 5 days. Only seeds with a hypocotyl protruding were counted as a sprout, and ruptured or swollen seeds were not counted. The germination rate was determined as the proportion of sprouted seeds to the total number of seeds. Experiments were performed in triplicate.

Scanning electron microscopy (SEM) analysis. Inoculated alfalfa and radish seeds were fixed in 2% Karnovsky's fixative for 2 h and washed three times with 0.05 M sodium cacodylate buffer for 10 min each. Seeds were immersed in a solution of 2% osmium tetroxide mixed with 0.1 M cacodylate buffer (1:1 v/v) for 2 h for post-fixation, and briefly washed twice with distilled water. The fixed alfalfa and radish seeds were dehydrated in a graded ethanol series (once in 30, 50, 70, 80, 90%, and three times in 100%) for 10 min each, and then completely dried in a Balzers CPD 030 critical point drying apparatus (BAL-TEC, Balzers, Liechtenstein). Dried seeds were mounted on aluminum stubs and then sputter-coated with gold using a vacuum coater (EM ACE200, Leica, Germany). Photomicrographs were observed using a Field-Emission Scanning Electron Microscope (SIGMA, Carl Zeiss, Germany).

Statistical analysis. All experiments were replicated three times. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan’s multiple range test at a probability level of $p < 0.05$.

III-3.3. Results

Effects of sequential treatment with ClO_2 gas and dry heat on populations of *E. coli* O157:H7 and *S.* Typhimurium. Dry heat treatment at 70 ℃ for 1 h caused significant ($p < 0.05$) log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, but thereafter, no further significant ($p > 0.05$) reductions occurred during 5h treatment (Table III-17). Dry heat treatment at 70 ℃ for 5 h resulted in 2.39 and 1.82 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Exposure to 150 ppmv of ClO_2 gas for 1 h resulted in 1.22 and 1.45 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Subsequent dry heat treatment (70 ℃) for 5 h caused 4.17 and 3.70 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Dry heat treatment at 80 ℃ for 5 h resulted in 3.08 and 3.23 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, respectively (Table III-18). ClO_2 gas treatment followed by dry heat treatment (80 ℃) for 5 h caused more than 5.32 and 5.29 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively.

Dry heat treatment at 70 ℃ was less effective at reducing populations of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds compared to alfalfa seeds, showing 1.72 and 1.43 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, after 5 h treatment (Table III-19). Sequential treatment with ClO_2 gas and dry heat treatment caused 3.33 and 2.69 log reductions of *E. coli* O157:H7 and *S.*

Typhimurium, respectively. Dry heat treatment at 80 ℃ for 5 h resulted in 2.49 and 2.27 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively (Table III-20). ClO_2 gas treatment followed by dry heat treatment (80 ℃) for 5 h caused 4.38 and 4.11 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively.

As dry heat treatment time increased, sequential treatment with ClO_2 gas and dry heat did not produce sublethally injured cells of *E. coli* O157:H7 and *S.* Typhimurium while dry heat treatment alone produced significant ($p < 0.05$) levels of injured cells of *E. coli* O157:H7 and *S.* Typhimurium.

Table III-17. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (70 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	-0.05±0.07Aa[b]	1.93±0.33Bb	2.12±0.43Ba	2.39±0.47Ba
		SPRAB	-0.07±0.27Aa	0.98±0.10Ba	1.47±0.41BCa	1.80±0.36Ca
	Dry heat + ClO_2 gas	SMAC	1.22±0.39Ab	3.03±0.15Bc	3.56±0.32BCc	4.17±0.49Cb
		SPRAB	1.04±0.41Ab	2.79±0.10Bc	2.86±0.28Bb	3.84±0.50Cb
S. Typhimurium	Dry heat alone	XLD	0.19±0.19Aa	1.46±0.47Bb	1.71±0.33Bb	1.82±0.36Bb
		OV-XLD	0.07±0.54Aa	0.56±0.42ABa	0.83±0.24ABa	1.05±0.49Ba
	Dry heat + ClO_2 gas	XLD	1.45±0.58Ab	2.66±0.41Bc	3.47±0.21Cd	3.70±0.21Cc
		OV-XLD	1.12±0.42Ab	1.94±0.23Bbc	2.77±0.40Cc	3.31±0.28Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-18. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (80 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.20±0.26Aa[b]	2.23±0.48Bb	2.51±0.13BCb	3.08±0.35Cb
		SPRAB	0.13±0.15Aa	1.38±0.16Ba	1.45±0.32BCa	1.76±0.02Ca
	Dry heat + ClO_2 gas	SMAC	1.58±0.52Ab	4.51±0.18Bc	5.25±0.08Cd	> 5.32Dc
		SPRAB	1.37±0.18Ab	4.00±0.26Bc	4.65±0.46Cc	> 5.32Dc
S. Typhimurium	Dry heat alone	XLD	0.38±0.28Aab	1.78±0.23Bb	2.71±0.25Cb	3.23±0.22Db
		OV-XLD	0.03±0.39Aa	1.27±0.14Ba	1.56±0.35BCa	2.15±0.40Ca
	Dry heat + ClO_2 gas	XLD	1.61±0.42Ac	4.46±0.15Bd	5.03±0.15Cd	> 5.29Dc
		OV-XLD	1.04±0.31Abc	3.68±0.29Bc	4.19±0.07Cc	> 5.29Dc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-19. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (70 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.08±0.12Aa[b]	0.94±0.41Ba	1.27±0.50BCab	1.72±0.28Cb
		SPRAB	0.06±0.07Aa	0.77±0.24Ba	0.97±0.57Ba	1.11±0.12Ba
	Dry heat + ClO_2 gas	SMAC	0.82±0.31Ab	2.18±0.05Bc	2.55±0.34Bc	3.33±0.24Cc
		SPRAB	0.33±0.15Aa	1.51±0.32Bb	1.83±0.10Bbc	2.87±0.26Cc
S. Typhimurium	Dry heat alone	XLD	0.01±0.16Aa	0.85±0.20Ba	1.11±0.46BCab	1.43±0.25Cb
		OV-XLD	0.17±0.41Aa	0.51±0.39ABa	0.62±0.25ABa	0.94±0.24Ba
	Dry heat + ClO_2 gas	XLD	0.90±0.24Ab	2.03±0.09Bc	2.52±0.08Cc	2.69±0.13Cc
		OV-XLD	0.47±0.30Aab	1.40±0.25Bb	1.66±0.28BCb	2.20±0.32Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Table III-20. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on radish seeds sequentially treated with 150 ppmv ClO_2 gas and dry heat (80 °C).

Microorganism	Treatment	Medium	Log reduction (log CFU/g)			
			Dry heat time (h)			
			0 h	1 h	3 h	5 h
E. coli O157:H7	Dry heat alone	SMAC	0.24±0.16Aab[b]	1.09±0.28Ba	1.81±0.44Ca	2.49±0.46Ca
		SPRAB	0.12±0.14Aa	1.34±0.30Ba	1.72±0.37Ba	1.95±0.47Ba
	Dry heat + ClO_2 gas	SMAC	0.91±0.01Ac	3.34±0.06Bb	4.12±0.14Cc	4.38±0.36Cb
		SPRAB	0.57±0.29Ab	2.87±0.31Bb	3.13±0.44Bb	3.82±0.31Bb
S. Typhimurium	Dry heat alone	XLD	0.06±0.08Aa	1.00±0.40Ba	1.54±0.12Ca	2.27±0.31Db
		OV-XLD	-0.08±0.06Aa	0.77±0.10Ba	1.11±0.38BCa	1.48±0.06Ca
	Dry heat + ClO_2 gas	XLD	1.16±0.25Ac	3.41±0.13Bc	3.69±0.10BCc	4.11±0.28Cc
		OV-XLD	0.77±0.21Ab	2.63±0.32Bb	2.77±0.34BCb	3.60±0.28Cc

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a row are significantly different ($p < 0.05$). For each pathogen species, means with different lowercase letters within a column are significantly different ($p < 0.05$).

Germination rate of seeds after sequential treatment with ClO_2 gas and dry heat. The germination rates of alfalfa and radish seeds after sequential treatments with ClO_2 gas and dry heat are shown in Table III-21. The germination rates of alfalfa seeds treated with dry heat alone (70 and 80 ℃) or sequential treatment with ClO_2 gas and dry heat (70 and 80 ℃) were not significantly ($p > 0.05$) different from those of untreated seeds. Also, the germination rates of radish seeds after all treatments except for sequential treatment with ClO_2 gas and dry heat (80 ℃) for 5 h were not significantly ($p > 0.05$) different from that of untreated seeds. The germination rate of radish seeds sequentially treated with ClO_2 gas and dry heat (80 ℃) decreased to 86.4% which was significantly ($p < 0.05$) different from that of untreated seeds.

Table III-21. Effect of sequential treatment with 150 ppmv ClO_2 gas and dry heat on germination of alfalfa and radish seeds.

Seed type	Treatment	Temperature (℃)	Germination rate (%)			
			Dry heat time (h)			
			0	1	3	5
Alfalfa	Dry heat alone	70	94.0±1.6A[a]	94.3±4.2A	93.9±3.1A	93.5±1.4A
	Dry heat + ClO_2 gas		92.8±0.9A	91.8±2.8A	93.4±5.0A	92.9±2.7A
	Dry heat alone	80	92.9±1.3A	93.2±1.4A	93.6±2.1A	93.5±2.3A
	Dry heat + ClO_2 gas		94.0±2.6A	93.1±2.5A	92.0±3.5A	94.1±2.8A
Radish	Dry heat alone	70	95.6±3.5A	93.7±4.7A	93.5±4.6A	94.5±3.0A
	Dry heat + ClO_2 gas		93.7±2.6A	94.4±3.9A	93.9±1.5A	94.8±1.3A
	Dry heat alone	80	92.4±1.2A	93.4±3.7A	94.1±2.5A	93.0±1.3A
	Dry heat + ClO_2 gas		93.6±2.8A	92.8±1.5A	93.6±3.1A	86.4±0.7B

[a] Values represent means and standard deviations of three times replications. Within the same row and temperature, different uppercase letters indicate significant differences ($p < 0.05$).

SEM image analysis. As shown in the SEM images (Fig. III-5), alfalfa seeds had smoother surfaces than radish seeds. Alfalfa seed surfaces consisted of shallow hills and valleys (Fig. III-5A). On the other hand, radish seed surfaces contained large and deep grooves surrounded by narrow hills (Fig. III-5B).

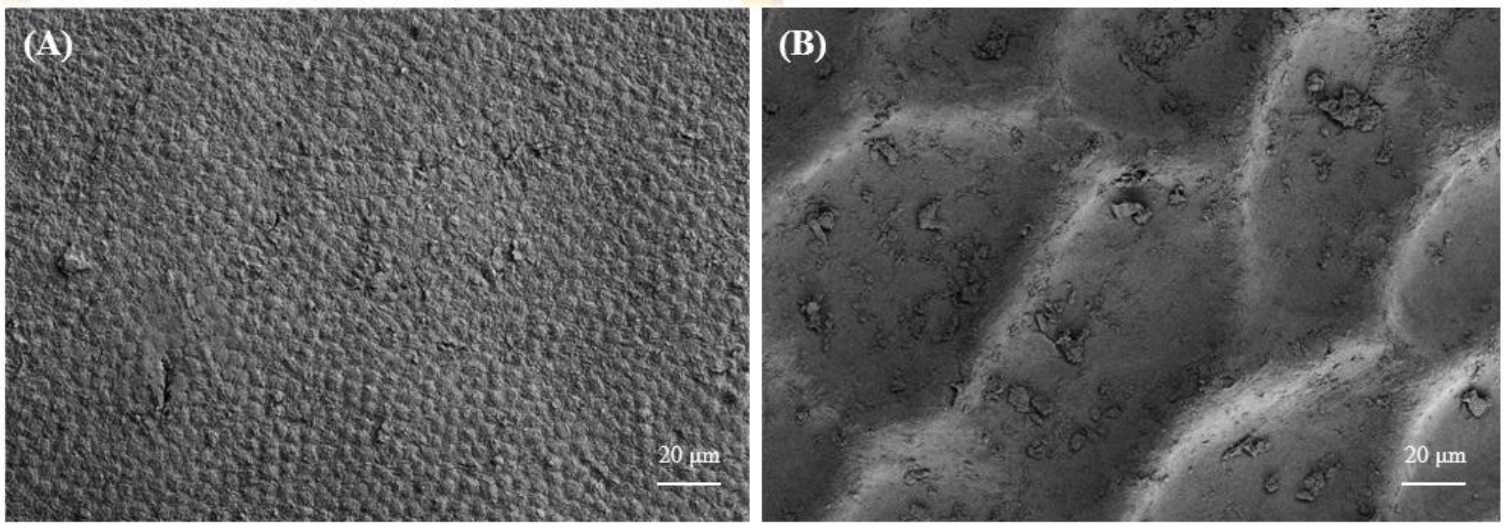


Fig. III-5. Scanning electron microscopy images of alfalfa (A) and radish (B) seeds.

III-3.4. Discussion

The FDA recommends achieving a 5-log reduction of pathogens on seeds used for sprout production (NACMCF, 1999). In the present study, 150 ppmv of ClO_2 gas treatment for 1 h resulted in 1.22 to 1.61 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds, and 0.90 to 1.16 log reductions on radish seeds. Annous and Burke (2015) reported exposure to ClO_2 gas (2 mg/L) for 6 h resulted in only 1.5 log reduction of *Salmonella* Montevideo on mung bean seeds. This means that ClO_2 gas treatment alone may be inadequate for controlling foodborne pathogens on seeds. Also, *E. coli* O157:H7 and *S.* Typhimurium populations on alfalfa or radish seeds decreased by 1.43 to 2.39 CFU/g after 5 h dry heat treatment at 70 ℃ for 5 h, and 2.27 to 3.23 log CFU/g after 5 h dry heat treatment at 80 ℃ for 5 h. Although dry heat treatment at 80 ℃ for 5 h achieved the greatest reductions in levels of *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds (3.08 and 3.23 log CFU/g, respectively), further reduction is required to meet the demands of the US FDA.

To achieve greater reductions in pathogen populations on seeds, combining two or more types of treatment, either simultaneously or in sequence, has been evaluated previously (Bang et al., 2011a; Bari et al., 2009; Kim et al., 2010; Neetoo et al., 2009; Neetoo and Chen, 2011). Annous and Burke (2015) evaluated the efficacy of the simultaneous application of ClO_2 gas and dry heat for reducing populations of *S.*

Montevideo populations on mung bean seeds. Dry heat (at 70 ℃ and 8% RH) for 4 h reduced *S.* Montevideo populations on mung bean seeds by 4.07 log CFU/g, and 4.77 log reduction was achieved when combination-treated with ClO_2 gas (3.5 mg/L). Only a marginal combination effect, possibly due to low RH (8% RH), occurred during combination treatment of ClO_2 gas and dry heat. It is well known that the antimicrobial effect of ClO_2 gas increases with increasing RH (Han et al., 2001a). In the present study, sequential treatments with ClO_2 gas and dry heat were applied to inactivate *E. coli* O157:H7 and *S.* Typhimurium on alfalfa and radish seeds. Sequential treatments with ClO_2 gas and dry heat showed additive or synergistic effects on microbial reductions: the total microbial inactivation of the combined treatment was not significantly ($p > 0.05$) different from or significantly ($p < 0.05$) higher than the sum of individual treatments. Oxidative attack on cell membrane proteins and enzyme and increased permeability of the outer cell membrane is the most widely accepted antimicrobial mechanism of ClO_2 (Aieta and Berg, 1986; Roller et al., 1980). Protein (structural and functional proteins) denaturation is a prime target for heat treatment. Also, heat treatment produces damage to the cell membrane, thereby altering the membrane permeability of bacteria (Russell, 2003). It seems that ClO_2 gas treatment sublethally damaged pathogen cells, thus their sensitivity to the following dry heat treatment increased. Ryu and Beuchat (2005) reported that ClO_2 treatment reduced heat resistance of

Bacillus cereus spores by damaging them. Heat resistance of *E. coli* O157:H7 was reduced after exposure to aqueous ClO_2 treatment (Kim et al., 2010a).

Bang et al. (2011c) evaluated the lethality of sequential treatments with aqueous ClO_2 and dry heat on microbial populations on radish seeds. *E. coli* O157:H7 on radish seeds treated with 500ug/ml aqueous ClO_2 for 5 min and subsequently heated at 60 ℃ (at 23% RH) for 48 h decreased by more than 4.8 log CFU/g. However, the germination percentage decreased from 91.2 to 68.7% after this sequential treatment. Dry heat treatment could damage aqueous ClO_2-treated radish seeds which have high moisture content, resulting in a reduced germination rate. To preserve seed viability, a drying interval between aqueous ClO_2 and dry heat treatment has been introduced (Bang et al., 2011a). Sequential treatments with aqueous ClO_2 (500 ug/ml) for 5 min, drying (45 ℃, 23% RH) for 24 h, and subsequent heating at 70 ℃ for 48 h eliminated *E. coli* O157:H7 on radish seeds without decreasing the germination rate. Although this sequential treatment effectively inactivated pathogens on seeds without negatively impacting seed germination, a long drying time is required. Conversely, a long drying interval is not required between ClO_2 gas and dry heat treatment because ClO_2 gas treatment does not greatly increase moisture content of seeds as does aqueous ClO_2. When inoculated alfalfa and radish seeds were dried in a laminar flow hood for 18 h, their moisture contents were 6.76 and 6.97%, respectively. After ClO_2 gas treatment for 1 h at 90% RH, those levels increased slightly to 7.76 and 8.11%.

Single as well as sequential treatment with ClO_2 gas and dry heat was more effective in reducing *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds than those pathogens on radish seeds. Differences in pathogen reduction levels may be due to differences in surface characteristics of seeds. Bari et al. (2009) reported that dry heat treatment at 50 ℃ for 17 h could reduce numbers of *E. coli* O157:H7 on alfalfa seeds to below the detection limit, while a 24 h treatment was required for radish seeds. Fransisca and Feng (2012) found that several sanitizers including $Ca(OCl)_2$ and malic acid reduced *E. coli* O157:H7 87-23 more on radish seeds than on alfalfa seeds. They reported the R_a (arithmetic mean roughness) value of radish seeds (6.08 um) was higher than that of alfalfa seeds (0.56 um), and generally a negative correlation existed between the R_a values of seeds and microbial reduction by sanitizer treatment. This means the more rough the seed surface, the less pathogen reduction occurred. As shown in Fig. II-5, alfalfa seeds had a smoother surface than radish seeds.

In conclusion, greater reductions were achieved by sequential treatment with ClO_2 gas and dry heat with short treatment times than those achieved by each single treatment. In the case of alfalfa seeds, 5-log reduction was achieved without decreasing the germination rate. Also, seed type could affect the efficacy of sequential treatment with ClO_2 gas and dry heat. Although ClO_2 gas and dry heat sequential treatment failed to completely inactivate *E. coli* O157:H7 and *S.*

Typhimurium on alfalfa and radish seeds, these treatments might be a potential commercial intervention that could be optimized.

Chapter IV.

Development of portable sustained-release formulation of ClO_2 gas for field application

IV-1. Introduction

ClO_2, a strong oxidizing agent, is an emerging technology for decontamination of fresh produce and food contact surfaces. ClO_2 has a broad antimicrobial spectrum and its efficacy is not largely affected by pH and organic matter (Aieta et al., 1984; Trinetta et al., 2012). The US EPA approved the use of aqueous chlorine dioxide for fruit and vegetable washing, meat and poultry disinfection, and food processing equipment sanitation. Also, ClO_2 gas has been approved for use in manufacturing and for sanitizing laboratory equipment, environmental surfaces, tools, and clean rooms (EPA, 2015). The US FDA approved the use of ClO_2 as an antimicrobial agent in water used in poultry processing and for washing fruits and vegetables (FDA, 1998). The antimicrobial effect of ClO_2 gas has been evaluated against foodborne pathogens on produce such as spinach (Neal et al., 2012; Park and Kang, 2015), potatoes (Wu and Rioux, 2010), apples (Du et al., 2002), tomatoes (Trinetta et al., 2013), lettuce (Mahmoud and Linton, 2008), mung bean sprouts (Prodduk et al., 2014), cabbage (Sy et al., 2005), cantaloupe (Mahmoud et al., 2008), and strawberries (Han et al., 2004). Also, the antimicrobial effect of ClO_2 gas against foodborne pathogens on food contact surfaces such as wood, plastic (Han et al., 2003), stainless steel (Vaid et al., 2010; Trinetta et al., 2012), polyvinyl chloride, and glass (Li et al., 2012; Morino et al., 2011) has been evaluated.

In spite of its antimicrobial effect and several advantages over aqueous sanitizers, there are some factors limiting widespread use of ClO_2 gas in disinfection activities (Stubblefield and Newsome, 2015). ClO_2 gas should be prepared at the application site due to its instability in shipment. Also, generation of ClO_2 gas typically entails the use of complicated equipment and trained personnel (Stubblefield and Newsome, 2015). Although several methods for on-site generation of ClO_2 gas without equipment have been developed (Fred, 2002; Isaac and Tenney, 2014; Yang and Kim, 2005; Engelhard Corporation, 2001), there are no suitable products for food application. ClO_2 gas sachets have been applied for the inactivation of foodborne pathogens on foods (Sy et al., 2005; Popa et al., 2007; Wu et al, 2010). However, these sachets release too much ClO_2 gas in a short time and it is difficult to control the generation rate of ClO_2 gas.

The objective of this study was to develop a mixture composition which could constantly release a low concentration of ClO_2 gas suitable for field application. Also, the antimicrobial effect of ClO_2 gas generated by this mixture against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on spinach leaves and tomatoes was evaluated and compared to ClO_2 gas generated by a lab scale ClO_2 gas generating system (chapter II-1).

IV-2. Materials and Methods

Development of mixture composition for ClO_2 gas generation. Sodium chlorite ($NaClO_2$), citric acid, and diatomaceous earth (DE) were purchased from Samchun Chemical Co. Ltd. (Pyeongtaek-si, South Korea). Calcium chloride dihydrate ($CaCl_2$) was purchased from Junsei chemical Co. Ltd. (Tokyo, Japan). The mixture compositions used in this study are shown in Table IV-1.

Table IV-1. Mixture composition for generating ClO_2 gas at 50 and 90% RH.

Mixture No.	RH condition	Composition (g)			
		$NaClO_2$	Citric acid	Diatomaceous earth	$CaCl_2$
1	50%	0.25	0.14	-	-
2		0.25	0.14	0.5	-
3		0.25	0.14	0.35	-
4		0.25	0.14	0.5	0.05
5		0.25	0.14	0.35	0.05
6	90%	0.25	0.14	-	-
7		0.25	0.14	3	-
8		0.25	0.14	6	-
9		0.25	0.14	7.5	-
10		0.25	0.14	9	-
11		0.25	0.14	12	-

Release profile of ClO_2 gas from mixtures as affected by conditions of relative humidity (RH). RH levels within the chamber were maintained by using 1 L of saturated aqueous salt solutions. Magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$) was purchased from Samchun chemical Co. Ltd. and sodium carbonate decahydrate ($Na_2CO_3 \cdot 10H_2O$) was purchased from Junsei chemical Co. Ltd., and used to create 50 and 90% RH, respectively. Petri dishes (90 × 15 mm; SPL Life Sciences, South Korea) containing thoroughly combined ClO_2 gas generation mixtures described above were placed in the treatment chamber (length × width × height, 0.7 m × 0.5 m × 0.6 m) in which the RH had been adjusted to 50 ± 2 or 90 ± 2% RH. ClO_2 gas concentration in the treatment chamber was observed for up to 36 h at 22 ± 1 ℃. A thermohygrometer (SE-342, Center Technology Corp., Taiwan) was used to record RH and temperature in the treatment chamber.

Bacterial cultures and cell suspension. Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890) and *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104) were obtained from the bacterial culture collection of the Food Safety Engineering Laboratory at Seoul National University (SNCC; Seoul, South Korea). Each strain (maintained as -80 ℃ frozen stocks) was streaked for isolation onto tryptic soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) and incubated for 24 h at 37 ℃. A single colony of each strain was inoculated into 5 ml of tryptic soy broth (TSB; Difco), incubated for 24 h at 37 ℃, collected by centrifugation at 4000 × g for 20 min at 4 ℃, and washed three times with buffered

peptone water (BPW; Difco). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7–10^8 CFU/ml. Suspended pellets of the two pathogens were combined to comprise a mixed culture cocktail containing approximately equal numbers of cells of each strain of *E. coli* O157:H7 and *S.* Typhimurium.

Sample inoculation. Spinach and tomatoes were purchased from a local market (Seoul, South Korea) and washed in running water and dried in a laminar flow biosafety hood (22 ± 2 ℃) for 1 h before experiments. Spinach leaves were trimmed to approximately 5 × 3 cm in size and the outer surface of tomatoes was cut into 5 × 2 cm pieces. Prepared spinach leaves and tomato surfaces were placed on aluminum foil in a laminar flow biosafety hood, and 0.1 ml of culture cocktail was inoculated onto one side of the sample by depositing droplets with a micropipettor at 15–20 locations. After inoculation, samples were dried in the biosafety hood at 22 ± 2 ℃ for 1 h with the fan running.

Inactivation of foodborne pathogens using ClO_2 gas generating mixture. ClO_2 gas was generated by mixtures No. 4 and 7 (Table III-1) under conditions of 50 and 90% RH, respectively. When the desired ClO_2 gas concentration was achieved, prepared samples were put into the treatment chamber. Samples were treated with 10, 20, or 30 ppmv of ClO_2 gas for up to 20 min at 50 or 90% RH at 22 ± 1 ℃. These experiments were repeated three times.

Bacterial enumeration. For microbial enumeration, treated spinach leaves (10 ± 0.2 g) and one piece of tomato were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 or 30 ml of neutralizing buffer (Difco), respectively. Stomacher bags were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min, and 1 ml aliquots withdrawn from stomacher bags were tenfold serially diluted in BPW, and 0.1 ml of appropriate diluents were spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco) and Xylose Lysine Desoxycholate agar (XLD; Difco) were used as selective media for enumeration of *E. coli* O157:H7 and *S.* Typhimurium, respectively. Where low levels of surviving cells were expected, 1 ml aliquots withdrawn from stomacher bags were divided between four plates of each medium and spread-plated to lower the detection limit. Plates were incubated at 37 ℃ for 24–48 h. Colonies were counted after incubation and calculated as log CFU/g and log CFU/cm^2 for spinach leaves and tomatoes, respectively.

Statistical analysis. All experiments were repeated three times. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA), and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

IV-3. Results

Release of ClO_2 gas generated from mixture as affected by RH. Fig. IV-1 shows the ClO_2 gas concentration released from mixtures at 50% RH for up to 36 h. A mixture containing only $NaClO_2$ and citric acid released 27 ppmv ClO_2 gas within ca. 6.5 h and ClO_2 gas concentration gradually decreased to 16 ppmv after 36 h. Addition of 0.5 and 0.35 g of DE reduced the maximum concentration of ClO_2 gas to 7 and 14 ppmv, respectively. ClO_2 gas released from these mixtures slightly decreased to 4 and 11 ppmv, respectively, after 36 h. Addition of $CaCl_2$ to gas generation mixtures facilitated a more constant release of ClO_2 gas. When 0.05 g of $CaCl_2$ was added to a mixture containing 0.5 g of DE, ClO_2 gas concentration remained relatively constant (11 ± 1 ppmv) from ca. 9.8 to 36 h. When 0.05 g of $CaCl_2$ was added to a mixture containing 0.35 g of DE, ClO_2 gas concentration remained at 16 ± 1 ppmv from ca. 12.5 to 36 h. Fig. IV-2 shows the ClO_2 gas concentration released from mixtures at 90% RH for up to 36 h. ClO_2 gas released from a mixture containing only $NaClO_2$ and citric acid reached 31 ppmv within ca. 3.2 h and then rapidly decreased to 3 ppmv after 36 h. Addition of DE to this mixture delayed the rate of ClO_2 gas generation. When 3 and 6 g of DE was added to mixture, ClO_2 gas concentration reached 30 ppmv within 7.8 and 12.5 h, respectively. Also, the decreasing rate of ClO_2 gas was delayed by addition of 3 and 6 g of DE. ClO_2 gas concentration after 36 h was 8 and 19 ppmv, respectively. ClO_2

gas released from a mixture containing 7.5 g of DE reached 30 ppmv within ca. 13.2 h and gradually decreased to 22 ppmv after 36 h. When 9 g of DE was added to this mixture, ClO_2 gas concentration remained relatively constant (26 ± 1 ppmv) from ca. 13.2 h to 36 h. Maximum ClO_2 gas concentration dropped to 19 ppmv with a mixture containing 12 g of DE, but ClO_2 gas concentration remained constant (18 ± 1 ppmv) from ca. 7.8 h to 36 h.

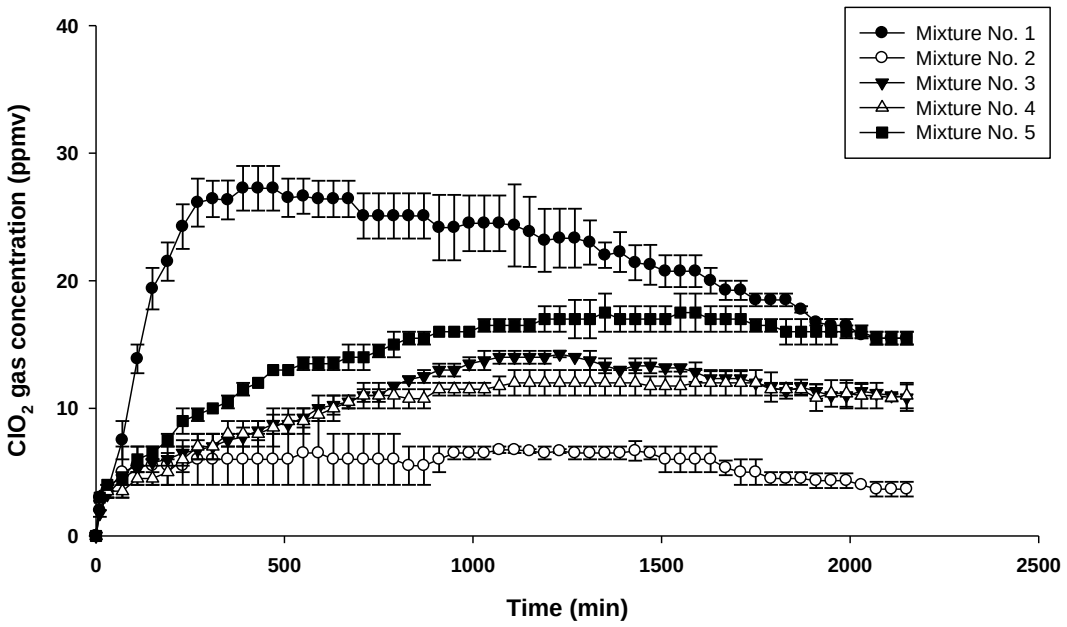


Fig IV-1. ClO_2 gas concentration released from mixtures into the treatment chamber at 50% RH for up to 36 h. The test was replicated at least three times.

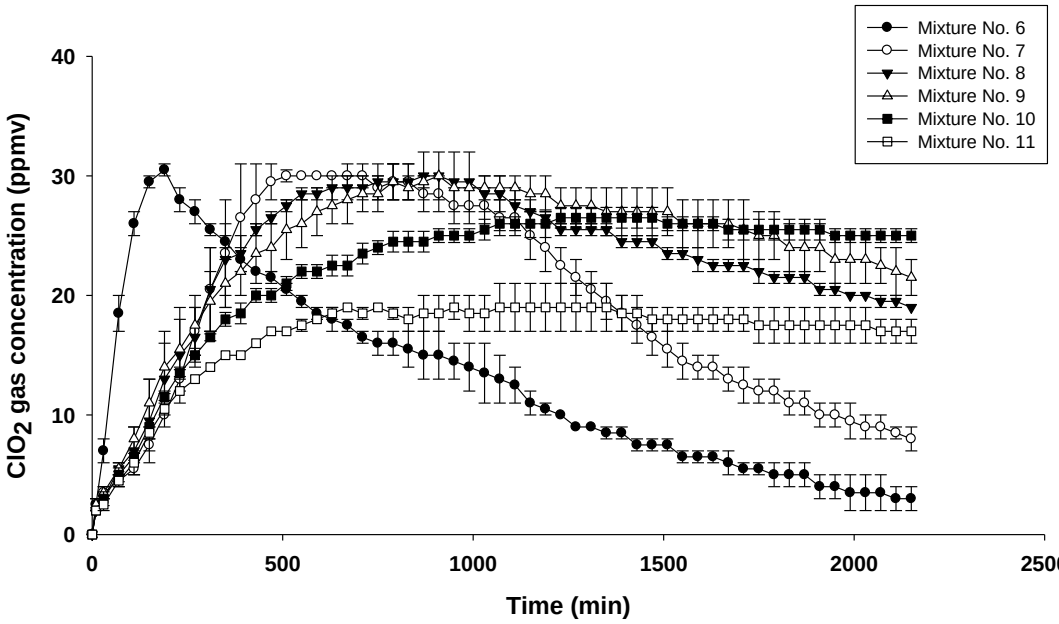


Fig IV-2. ClO_2 gas concentration released from mixtures into the treatment chamber at 90% RH for up to 36 h. The test was replicated at least three times.

Inactivation of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes by ClO_2 gas. The antimicrobial effects of ClO_2 gas generated from mixture against *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes are shown in Tables IV-2 to 8. At 50% RH, exposure to 10 ppmv of ClO_2 gas generated from mixture for 20 min caused 1.02 and 1.16 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves, and 1.02 and 1.14 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes, respectively (Table IV-2 and 3). There were no significant ($p > 0.05$) differences between log reductions obtained by lab scale and mixture type ClO_2 gas generation.

Treatment with 10 ppmv of mixture-generated ClO_2 gas for 20 min resulted in 3.76 and 3.39 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves at 90% RH (Table IV-4). More than 6.16 and 5.48 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves were observed after treatment with 30 ppmv of ClO_2 gas generated by the mixture at 90% RH for 15 min (Table IV-5). Exposure to 10 ppmv of mixture-generated ClO_2 gas at 90% RH for 20 min caused 4.38 and 4.39 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, on tomatoes (Table IV-6). Treatment with 20 ppmv of ClO_2 gas for 15 min caused more than 6.99 and 6.73 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively (Table IV-7). After treatment with 30 ppmv of ClO_2 gas for 10 min, more than 6.78 and 6.34 log reductions of *E. coli* O157:H7 and *S.* Typhimurium, respectively, were observed (Table IV-8). There were no significant ($p > 0.05$)

differences between log reductions obtained by lab scale and mixture type ClO_2 gas generation.

Table IV-2. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves after treatment with 10 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 50% RH.

Treatment time	Log reduction (log CFU/g)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
5 min	0.50±0.21A[b]	0.79±0.46A	0.57±0.52A	0.87±0.29A
10 min	0.91±0.13A	0.80±0.58A	0.67±0.18A	0.98±0.20A
15 min	1.14±0.31A	0.96±0.36A	0.76±0.19A	1.00±0.07A
20 min	1.32±0.22A	1.02±0.45A	1.20±0.21A	1.16±0.05A

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-3. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes after treatment with 10 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 50% RH.

Treatment time	Log reduction (log CFU/cm^2)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
5 min	0.80±0.28A[b]	0.74±0.12A	0.82±0.35A	0.95±0.08A
10 min	0.87±0.25A	0.73±0.11A	1.11±0.38A	0.92±0.10A
15 min	1.07±0.26A	0.92±0.29A	1.22±0.36A	1.01±0.16A
20 min	1.10±0.24A	1.02±0.17A	1.33±0.42A	1.14±0.06A

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-4. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves after treatment with 10 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 90% RH.

Treatment time	Log reduction (log CFU/g)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
5 min	1.22±0.15A[b]	1.74±0.18B	1.08±0.54A	1.89±0.33A
10 min	2.51±0.32A	2.30±0.24A	2.32±0.20A	2.48±0.35A
15 min	3.08±0.41A	2.72±0.22A	2.94±0.23A	2.93±0.32A
20 min	3.63±0.37A	3.76±0.49A	3.56±0.51A	3.39±0.10A

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-5. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves after treatment with 30 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 90% RH.

Treatment time	Log reduction (log CFU/g)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
1 min	1.06±0.43A[b]	1.35±0.17A	0.98±0.10A	1.56±0.17B
5 min	1.99±0.37A	2.06±0.06A	1.69±0.08A	1.96±0.11B
10 min	4.02±0.50A	4.39±0.11A	3.76±0.32A	3.54±0.59A
15 min	> 5.78A	> 6.16A	> 5.68A	> 5.48A

[a] Log reduction = population (log CFU/g) before treatment – population (log CFU/g) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-6. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes after treatment with 10 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 90% RH.

Treatment time	Log reduction (log CFU/cm^2)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
5 min	1.56±0.42A[b]	1.25±0.03A	1.36±0.32A	1.37±0.18A
10 min	2.34±0.32A	1.75±0.09B	1.92±0.36A	1.78±0.06A
15 min	2.98±0.25A	3.28±0.28A	2.61±0.24A	3.05±0.25A
20 min	4.26±0.65A	4.38±0.21A	4.33±0.40A	4.39±0.36A

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-7. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes after treatment with 20 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 90% RH.

Treatment time	Log reduction (log CFU/cm^2)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
1 min	0.45±0.15A[b]	0.91±0.42A	0.55±0.09A	0.72±0.53A
5 min	1.41±0.21A	1.37±0.11A	1.38±0.31A	1.51±0.12A
10 min	3.60±0.53A	3.37±0.17A	3.50±0.49A	3.68±0.18A
15 min	> 6.74A	> 6.99A	> 6.93A	> 6.73A

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

Table IV-8. Log reductions[a] of *E. coli* O157:H7 and *S.* Typhimurium on tomatoes after treatment with 30 ppmv ClO_2 gas generated by lab scale system and portable mixture type at 90% RH.

Treatment time	Log reduction (log CFU/cm^2)			
	E. coli O157:H7		*S.* Typhimurium	
	Lab scale system	Portable type	Lab scale system	Portable type
1 min	0.73±0.16A[b]	1.26±0.23B	0.89±0.03A	1.15±0.33A
5 min	2.30±0.38A	2.52±0.36A	2.02±0.18A	2.68±0.12B
10 min	> 6.85A	> 6.78A	> 6.90A	> 6.34A

[a] Log reduction = population (log CFU/cm^2) before treatment – population (log CFU/cm^2) after treatment.

[b] Means with different uppercase letters within a raw are significantly different ($p < 0.05$).

IV-4. Discussion

The release of ClO_2 gas from mixture was significantly affected by RH. ClO_2 gas was more rapidly released from mixtures containing only $NaClO_2$ and citric acid at 90% RH than at 50% RH. Also, ClO_2 gas concentration decreased more rapidly at 90% than at 50% RH. It seems that $NaClO_2$ and citric acid dissolved and thus reacted more rapidly at 90% RH due to greater treatment chamber moisture and rapidly released ClO_2 gas into the treatment chamber. To maintain a constant ClO_2 gas concentration in the treatment chamber, the release rate of ClO_2 gas should be slowed.

DE is a soft, friable, very fine-grained, siliceous sedimentary rock created by the deposition of fossilized single-cell algae on the ocean and fresh water floors (Janićijević et al., 2014). Frustules vary in size, shape and architecture depending on a diverse array and the species of diatoms, but basically they represent a highly porous, yet rigid, amorphous silica skeletal framework (Akhtar et al., 2009; Janićijević et al., 2014). DE is an attractive material owing to its low cost, well-defined porosity, low density, and has a broad spectrum of applications such as filters, drug delivery, and removal of heavy metals or pollutants (Arik, 2003; Caliskan et al., 2011; Janićijević et al., 2014; Martinovic et al., 2006; Zhang et al., 2011).

In the present study, DE was used to induce sustained release of ClO_2 gas. DE could absorb water from the chamber and gradually release it due to its highly porous structure, and this prevented a rapid reaction of $NaClO_2$ with citric acid. Also, DE could absorb generated ClO_2 gas and slowly release it to the chamber. At 90% RH, addition of DE to the mixture could control the generation rate and maximum concentration of ClO_2 gas. When 9 and 12 g of DE were added to the mixture, ClO_2 gas concentration remained constant at 26 ± 1 ppmv for ca. 23 h and at 18 ± 1 ppmv for ca. 28 h, respectively. At 50% RH, the generation rate and maximum concentration of ClO_2 gas also could be controlled by adding DE and more constant ClO_2 gas concentration was maintained by adding $CaCl_2$ to the mixture. When 0.05 g of $CaCl_2$ was added to mixtures containing 0.5 and 0.35 g of DE, ClO_2 gas concentration remained constant at 11 ± 1 ppmv for ca. 26 h and at 16 ± 1 ppmv for ca. 24 h, respectively. $CaCl_2$, a salt adsorbent, has greater hygroscopic capacity than organic adsorbents (Zhang and Qiu, 2007). It seems that $CaCl_2$ could facilitate a more constant reaction of $NaClO_2$ with citric acid under conditions of lower RH.

In the present study, ClO_2 gas generated by mixtures showed a significant antimicrobial effect against *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes. More than 6.16 and 5.48 log, and 6.78 and 6.34 log reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes were observed after treatment with 30 ppmv of ClO_2 gas generated by mixtures for 15 and 10 min, respectively, at 90% RH. Also, reductions of *E. coli* O157:H7 and *S.* Typhimurium

achieved by ClO_2 gas treatment using mixtures generally did not significantly ($p > 0.05$) differ from those achieved by a lab scale ClO_2 gas generation system (chapter II-1). This demonstrates that mixture-generated ClO_2 gas shows the same antimicrobial effect as ClO_2 gas generated by a lab scale system.

In conclusion, we developed portable ClO_2 gas generating mixtures for sustained release of ClO_2 gas. RH conditions affected the ClO_2 gas release profile, and the generation rate and maximum ClO_2 gas concentration could be controlled using DE and $CaCl_2$. ClO_2 gas produced by portable mixtures showed significantly the same reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes as ClO_2 gas generated by a lab scale system. The portable ClO_2 gas generating mixture of this study could facilitate the use of ClO_2 gas by the food industry, but further study is required to optimize packaging and utilization of the mixture for practical application such as food storage and food transportation.

Chapter V.

Overall conclusion

V-1. Overall results

ClO_2 gas showed significant antimicrobial effect against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on produce and food contact surfaces in the present study. This study showed several factors could affect the antimicrobial effect of ClO_2 gas. Significant differences ($p < 0.05$) were observed between inactivation levels under different RH conditions as ClO_2 gas concentration and treatment time increased. Variations in RH have great effect on the solubilization of ClO_2 gas on produce surfaces considering that ClO_2 concentration on those produce surfaces increased with increasing RH. Also, the amount of ClO_2 concentration on produce surfaces is correlated with the level of inactivation of pathogens. Surface characteristics of produce and food contact surfaces have a great impact on the inactivation of foodborne pathogens by ClO_2 gas treatment. The results of this study indicate that surface hydrophobicity plays a more important role in bacterial inactivation from surfaces than surface roughness. Contact angles of produce and food contact surfaces showed highly negative correlation with the log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*: that is, the more hydrophobic the surface, the less the reduction of the three pathogens. Treatment temperature also has a great impact on the inactivation of foodborne pathogens by ClO_2 gas treatment, and increased solubility of ClO_2 gas at low temperatures plays an important role in bacterial inactivation from surfaces. The results of this study are

helpful for the food industry to establish ClO_2 gas treatment conditions for maximizing the antimicrobial efficacy of ClO_2 gas.

This study suggested combination treatment of ClO_2 gas with other control methods to inactivate foodborne pathogens while lowering the concentration of ClO_2 gas. As treatment time increased the combination treatment of UVC and ClO_2 gas could show additive or synergistic effects in the inactivation of three foodborne pathogens depending on type of produce and ClO_2 gas concentration. The mechanism of the synergistic effect was related to membrane damage, followed by changes to membrane permeability. The combination treatment of ClO_2 gas and aerosolized PAA showed additive effects in the inactivation of three foodborne pathogens with inactivation generally superior to that of each treatment applied individually, as treatment time increased. Also, greater reductions were achieved by sequential treatment with ClO_2 gas and dry heat with short treatment times than those achieved by each single treatment. In the case of alfalfa seeds, 5-log reduction was achieved without decreasing the germination rate. The combination treatment of ClO_2 gas with other technology in this study may suggest alternatives to currently used decontamination methods.

For field application, this study developed portable ClO_2 gas generating mixtures for sustained release of ClO_2 gas. RH conditions affected the ClO_2 gas release profile, and the generation rate and maximum ClO_2 gas concentration could be controlled using DE and $CaCl_2$. ClO_2 gas produced by portable mixtures showed

significantly the same reductions of *E. coli* O157:H7 and *S.* Typhimurium on spinach leaves and tomatoes as ClO_2 gas generated by a lab scale system. Portable ClO_2 gas generating mixture could facilitate the use of ClO_2 gas in the food industry.

V-2. Suggestion for further study

The present study focused on evaluating inactivation tendency of foodborne pathogens by intrinsic and extrinsic factors, but further study is needed to define the processing conditions necessary to achieve a certain level of microbial safety. To achieve this goal, the kinetic models should be proposed to analyze available data, as well as to provide a basis for optimizing process conditions considering the effect of several factors that are important to the outcome of the inactivation of foodborne pathogens, including environmental factors, microbial characteristics, and produce characteristics.

In case of combination treatment studies, further study is needed to clarify additive or synergistic effect on the microbial inactivation by each combination treatment. Through inactivation mechanism study, it may be possible to enhance the antimicrobial efficacy of each combination treatment. In case of combination treatment of ClO_2 gas with aerosolization, it is needed to search sanitizers other than PAA which could represent synergistic antimicrobial effect when combined used with ClO_2 gas. Also, it would be interesting to investigate optimum food samples for each combination treatment.

The present study developed ClO_2 gas generating compositions for sustained release of ClO_2 gas, but further study is required to optimize packaging of the mixture for practical application such as food storage and food transportation.

Selection of packaging material of mixture is important because it could affect ClO_2 gas releasing profiles. Secondary control of ClO_2 gas release is possible using suitable packaging material. Also, using potable mixture which release low concentration of ClO_2 gas, long-term exposure experiment is needed to confirm sustained inhibition of the growth of foodborne pathogens. It is important for practical filed application.

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Chapter VI.

Appendix: Inactivation study of foodborne pathogens using other control methods

VI-1. Use of organic acids to inactivate *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce

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VI-1.1. Abstract

This study was undertaken to investigate the antimicrobial effect of organic acids against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on whole red organic apples and lettuce. Several studies have been conducted to evaluate organic acids as sanitizers. However, no studies have compared antimicrobial effects of various organic acids on organic fresh produce, including evaluation of color changes of produce. Apples and lettuce were inoculated with a cocktail of three strains each of three foodborne pathogens provided above and treated with 1 and 2% organic acids (propionic, acetic, lactic, malic, and citric acid) for 0, 0.5, 1, 5, and 10 min. With increasing treatment time and acid concentration, organic acid treatments showed significant reduction compared to the control treatment (distilled water), and differences in antimicrobial effects between organic acids were observed. After 10 min of treatment with 1 and 2% organic acids in apples, propionic (0.92 to 2.75 log reduction), acetic (0.52 to 2.78 log reduction), lactic (1.69 to >3.42 log reduction), malic (1.48 to >3.42 log reduction), and citric acid (1.52 to >3.42 log reduction) exhibited significant ($p < 0.05$) antibacterial effects against three foodborne pathogens compared to the control treatment. In lettuce, propionic (0.93 to 1.52 log reduction), acetic (1.13 to 1.74 log reduction), lactic (1.87 to 2.54 log reduction), malic (2.32 to 2.98 log reduction), and citric acid (1.85 to 2.86 log reduction) showed significant ($p < 0.05$) effects

compared to the control treatment. Changes in sample color subjected to organic acids treatment were not significant during storage.

VI-1.2. Introduction

Consumption of organic fresh fruits and vegetables has been increasing (Beuchat, 2002) as consumers become increasingly concerned about their health. In the United States, the organic food industry has been growing rapidly in the past two decades. It is estimated that organic sales have increased by nearly 20% annually since 1990, with consumer sales reaching $13.8 billion in 2005 (Carl and Davis, 2006).

However, foodborne disease outbreaks associated with fresh produce have been reported (Lang et al., 2004; Shearer et al., 2001). Among foodborne pathogens, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are of greatest public health concern (Burnett and Beuchat, 2001). These foodborne pathogens have been isolated from lettuce (CDPH, 2008; Cooley et al., 2007; Smith DeWaal et al., 2006), mixed fruits, prepared salad (Little and Gillespie, 2008; Sivapalasingam et al., 2004), spinach (CDPH, 2007; CDC, 2006), sprouts (Breuer et al., 2001; Harris et al., 2003), and tomatoes (CDC, 2005; Cummings et al., 2001)

Generally, washing with tap water is inadequate to control foodborne pathogens unless combined with effective sanitizers (Adams et al., 1989). Different sanitizers have varying degrees of antimicrobial effects on fresh produce. Chlorinated water, widely used, has little antimicrobial effect (less than 2 log reduction) (Cherry 1999; Taormina et al., 1999), and is known to react with organic matter resulting in the formation of carcinogenic halogenated by-products (Hua and Reckhow, 2007).

Many sanitizers, such as chlorine dioxide (ClO_2) (Beuchat, 1998), trisodium phosphate (Zhuang and Beuchat, 1996), hydrogen peroxide (H_2O_2) (Lin et al., 2002), and ozonated water (Restaino et al., 1995) have already been evaluated for their effective usage against pathogens.

However, in organic production fields, organically approved sanitizers are not sufficient. Although a limited number of synthetic sanitizers have been approved by the National Organic Standards Board (NOSB) and National Organic Program (NOP), the food industry is still seeking alternative sanitizers, such as organic acids to assure disinfection (Diez-Gonzalez and Feirtag, 2008; Ö lmez and Kretzschmar, 2009).

Organic acids are generally recognized as safe (GRAS) for use as a food ingredient. Some organic acids have been evaluated as sanitizers on fruits and vegetables. Antimicrobial activity of organic acids (lactic, citric, acetic, and ascorbic acid) against *E. coli* and *L. monocytogenes* was compared on iceberg lettuce (Akabas and Ö lmez, 2007). Zhang and Farber (1996) evaluated the combination effect of lactic and acetic acid with chlorine to reduce *L. monocytogenes* on shredded lettuce. Also, lactic acid with hydrogen peroxide treatment to reduce *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* on apples, oranges, and tomatoes was investigated (Venkitanarayanan et al., 2002). However, there have been no studies comparing antimicrobial effects of various organic acids on organic fresh produce.

This study evaluated the antimicrobial effects of five different organic acids (propionic, acetic, lactic, malic, and citric acid) against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh apples and lettuce. Also the effect of acid treatment on sample color was investigated.

VI-1.3. Materials and Methods

Bacterial strains. Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the School of Food Science bacterial culture collection, Washington State University (Pullman, WA, USA), for this study.

Culture preparation. One loop (10 µL) of frozen culture was transferred to 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) in a 20 ml culture tube. Static culture was maintained by daily transfer in TSB (37 ℃, 18 to 24 h incubation). Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured in 10 ml of TSB at 37 ℃ for 24 h, harvested by centrifugation at 4000 × g for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7-10^8 CFU/ml. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* isolates were combined to make culture cocktails for use in experiments.

Sample inoculation. Unwaxed whole, red apples and lettuce were purchased from a local market (Seoul, South Korea) on the day of experiments. Visible soil on apples was removed with sterile tissue paper and the outer leaves of lettuce were aseptically removed. Inner leaves were trimmed to 25 g using a sterile knife. Apples

and lettuce leaves were placed on aluminum foil in a laminar flow hood, and inoculated with 0.1 ml of culture cocktails by depositing droplets with a micropipet at 10-15 locations. After inoculation, samples were dried in a laminar flow biosafety hood for 3 h at 22 ± 2 ℃ to facilitate the attachment of bacteria, and subjected to organic acid treatments.

Preparation of organic acids. Organic acids; propionic acid (99%; Samchun Chemical Co. Ltd., Pyeongtaeksi, South Korea), acetic acid (99.7%; Junsei Chemical Co. Inc., Tokyo, Japan), malic acid (99%; Samchun Chemical), lactic acid (85.0-92.0%; Kanto Chemical Co. Inc., Tokyo, Japan), and citric acid (99.5%; Junsei Chemical) were used in this experiment. Propionic acid, acetic acid, lactic acid (1% and 2%, v/v); malic acid, citric acid (1% and 2%, w/v) were prepared (22 ± 2 ℃) using sterile distilled water. The pH for 1% and 2% organic acids is as follows: propionic acid: 2.81, 2.71; acetic acid: 2.75, 2.61; lactic acid: 2.26, 2.12; malic acid: 2.29, 2.17; citric acid: 2.25, 2.09.

Sample treatments with organic acids. Inoculated samples were immersed in either 1 L of 1% or 2% of each organic acid for 0, 0.5, 1, 5, and 10 min. Distilled water was also included as a control treatment. All experiments were performed at room temperature (22 ± 2 ℃).

Bacterial enumeration. The treated samples (whole apple and lettuce leaves) were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 50 ml of Dey-Engley (DE) neutralizing broth (Difco).

Stomacher bags were gently massaged for 1 min and then shaken for 1 min to dislodge pathogens from apples (Wisniewsky and others 2000). Stomacher bags containing lettuce leaves were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. After homogenization, DE broth was tenfold serially diluted in BPW, and 0.1 ml of sample or diluents was spread-plated onto selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), Oxford agar base with "Bacto^{TM} Oxford antimicrobic supplement" (MOX; Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. When low bacterial numbers were anticipated, 250 μl of sample was plated onto four plates of each respective medium. The plates were incubated at 37˚C for 24-48 h. Colonies were counted and calculated as log CFU/apple and log CFU/g in lettuce samples.

Color analysis. Colors of the samples were analyzed with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan). Water rinsing was performed for 15 s after organic acid treatment. Hunter's color values (L, a, b) were measured at 2 locations of each sample. In the case of apples, measurement was done in the reddest part of samples. Hunter's L, a, b values indicated lightness, redness, and yellowness of the sample, respectively.

Statistical analysis. All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC,

USA) and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

VI-1.4. Results

Inactivation of foodborne pathogens on apples and lettuce. The initial level of *E. coli* O157:H7 was 6.94 log CFU/apple (Table VI-1). After 0.5 min, the control treatment (distilled water) reduced the cell population to 4.83 log CFU/apple, but significant ($p < 0.05$) further reduction occurred following treatment with 1% propionic acid (PA) and citric acid (CA) (0.41 and 0.58 log CFU/apple, respectively), and with 2% acetic acid (AA), lactic acid (LA), malic acid (MA), and CA (0.52, 0.71, 0.76, and 0.75 log CFU/apple, respectively). Dramatic differences in reduction between the control and acid treatments were observed with increasing treatment time. After 1 min, *E. coli* O157:H7 was significantly reduced with 1% MA treatment (0.94 log CFU/apple) and with 2% PA, AA, LA, MA, and CA treatment (0.76, 0.75, 0.60, 0.95, and 1.51 log CFU/apple, respectively) compared to the control treatment. Levels of *E. coli* O157:H7 were significantly ($p < 0.05$) reduced by 0.46, 0.55, 0.64, 0.98, and 0.98 log CFU/apple after 5 min of treatment with 1% PA, AA, LA, MA, and CA, respectively, and treatment with 2% concentration of these acids reduced levels by 0.80, 1.27, 1.63, 1.77, and 2.27 log CFU/apple, respectively. More than 2.56 log reductions were observed with 2% LA and CA treatment after 10 min, compared to the control treatment.

The initial population of *S.* Typhimurium was 7.37 log CFU/apple (Table VI-2). Increasing the control treatment time did not significantly reduce levels of *S.*

Typhimurium. Further reduction occurred after 0.5 min of treatment with 1% PA, AA, LA, MA, and CA (1.22, 1.03, 1.69, 1.33, and 1.23 log CFU/apple, respectively) and with 2% of these organic acids (1.77, 1.12, 1.64, 1.33, and 1.99 log CFU/apple, respectively) compared to the control treatment. After 1 min, treatment with 1% PA, AA, LA, MA, and CA resulted in further reductions of 1.24, 0.92, 1.77, 1.16, and 1.18 log CFU/apple, respectively, and 2% concentration of these organic acids resulted in further reductions of 2.03, 1.48, 1.96, 1.33, and 1.90 log CFU/apple, respectively, compared to the control treatment. CA (2%) treatment exhibited >3.42 log reduction after 5 min, and LA, MA, and CA (2%) treatments showed >3.42 log reduction after 10 min, compared to the control treatment.

The initial level of *L. monocytogenes* was 7.26 log CFU/apple (Table VI-3). After 0.5 min, *L. monocytogenes* was significantly ($P < 0.05$) reduced following treatment with 1% MA and CA (0.86 and 0.99 log CFU/apple, respectively), and with 2% MA and CA (0.91 and 1.25 log CFU/apple, respectively) compared to the control treatment. When 1% organic acids were applied for 1 min, only CA showed a significant ($p < 0.05$) effect (further 0.62 log reduction) compared to the control treatment. Two percent PA, AA, LA, MA, and CA treatment resulted in significant ($P < 0.05$) further reductions below the control treatment of 1.13, 1.15, 1.11, 0.81, and 1.27 log CFU/apple, respectively. After 5 min, significant ($p < 0.05$) reductions occurred following treatment with 1% PA, AA, LA, MA, and CA (0.90, 2.10, 1.19, 2.70, and 1.76 log CFU/apple, respectively), and with 2% concentration of these

organic acids (1.74, 2.02, 2.44, 2.55, and 2.50 log CFU/apple, respectively) compared to the control treatment. More than 2.90 log reductions occurred when 1% LA and MA, 2% LA, MA, and CA were treated for 10 min.

The antibacterial effects of organic acids against the 3 tested pathogens on lettuce are shown in Table VI-4, 5, and 6. Generally, increasing the time interval of the control treatment had no significant effect on reducing levels of the three foodborne pathogens. The initial population of *E. coli* O157:H7 was 7.03 log CFU/g (Table VI-4). After 0.5 min treatment, 1% organic acids, except PA and AA, and 2% of PA, AA, LA, MA, and CA exhibited further reductions of 0.56, 0.85, and 0.58 log CFU/g (1%), and 0.65, 0.63, 0.82, 1.08, and 0.78 log CFU/g (2%) respectively, compared to the control treatment. The population of *E. coli* O157:H7 was reduced to below that of the control treatment after 1 min of exposure to 1% LA, MA, and CA (1.13, 0.92, and 0.99 log CFU/g, respectively), and 2% PA, AA, LA, MA, and CA (0.93, 0.81, 1.38, 1.22, and 1.34 log CFU/g, respectively). Levels of *E. coli* O157:H7 were significantly ($p < 0.05$) reduced by 1.29, 1.25, 2.04, 2.11, and 1.91 log CFU/g after 5 min of treatment with 1% PA, AA, LA, MA, and CA, respectively, and treatment with 2% concentration of these acids reduced levels by 1.56, 1.57, 2.63, 2.54, and 2.03 log CFU/g, respectively. After 10 min, the populations in all acid treatments were significantly ($p < 0.05$) reduced with 1% PA, AA, LA, MA, and CA treatment (1.20, 1.13, 2.04, 2.48, and 2.25 log CFU/g, respectively), and

with 2% concentration of these acids (1.52, 1.41, 2.43, 2.72, and 2.86 log CFU/g, respectively), compared to the control treatment.

The initial population of *S.* Typhimurium was 7.04 log CFU/g (Table VI-5). Significant ($p < 0.05$) reduction was observed after 0.5 min of treatment with 1% PA, AA, LA, and MA (0.80, 0.78, 0.68, and 1.06 log CFU/g, respectively), and with 2% levels of these acids (0.96, 1.01, 0.94, 1.19, and 1.50 log CFU/g, respectively), compared to the control treatment. Regarding 1 min treatment with 1% organic acids, MA and CA significantly ($p < 0.05$) reduced levels of *S.* Typhimurium by 0.92 and 0.89 log CFU/g, and 2% AA, LA, MA, and CA treatment reduced levels by 0.79, 0.94, 1.28, and 1.14 log CFU/g, respectively, compared to the control treatment. Greater reduction than that of the control occurred with 1% AA, LA, MA, and CA treatment (0.96, 1.66, 1.79, and 1.80 log CFU/g, respectively), and with 2% PA, AA, LA, MA, and CA treatment (1.12, 1.69, 1.93, 3.61, and 2.02 log CFU/g, respectively) after 5 min. Significant ($p < 0.05$) reductions following 10 min of treatment with 1% PA, AA, LA, MA, and CA occurred (0.93, 1.64, 1.87, 2.32, and 1.85 log CFU/g, respectively), and with 2% concentration of these organic acids (1.22, 1.74, 2.54, 2.98, and 2.32 log CFU/g, respectively) compared to the control treatment.

The initial level of *L. monocytogenes* was 6.45 log CFU/g (Table VI-6). After 0.5 min, 2% LA, MA, and CA treatment showed a significant ($p < 0.05$) effect (1.60, 1.62, and 1.20 log reductions, respectively), compared to the control treatment. After 1 min, further reduction occurred with 1% LA, MA, and CA treatment (1.35, 1.52,

and 1.23 log CFU/g, respectively) and with 2% of AA, LA, MA, and CA treatment (1.58, 2.02, 1.72, and 2.10 log CFU/g, respectively), compared to the control treatment. After 5 min, all acid treatments, except 1% PA, produced significant ($P < 0.05$) reductions compared to the control treatment, further reductions occurred with 1% AA, LA, MA, and CA treatment (0.91, 1.59, 1.95, and 1.30 log CFU/g, respectively), and with 2% PA, AA, LA, MA, and CA treatment (0.90, 1.56, 2.13, 2.50, and 2.08 log CFU/g, respectively). After 10 min, the population was significantly ($p < 0.05$) reduced with 1% PA, AA, LA, MA, and CA treatment (1.01, 1.37, 2.21, 2.60, and 2.06 log CFU/g, respectively), and with 2% concentration of these acids (1.18, 1.69, 2.48, 2.96, and 2.42 log CFU/g, respectively), compared to the control treatment.

Table VI-1. Effect of organic acid treatment on inactivation of *E. coli* O157:H7 on whole, red, unwaxed apples.

Treatment (Organic acid)	Number of *E. coli* O157:H7 (log CFU/apple)[a]			
	0.5 min	1 min	5 min	10 min
DW	4.83±0.15Aa[b]	4.58±0.20ABa	4.45±0.27ABa	4.26±0.31Ba
Propionic acid (1%)	4.42±0.52Abc	4.27±0.41Aab	3.99±0.29ABb	3.34±1.04Bbc
Propionic acid (2%)	4.49±0.47Aabc	3.82±0.17Abc	3.65±0.17ABbcd	3.21±0.71Bbc
Acetic acid (1%)	4.59±0.20Aab	4.64±0.35Aa	3.90±0.10Bb	3.74±0.12Bab
Acetic acid (2%)	4.31±0.23Abc	3.83±0.47ABbc	3.18±0.15BCde	2.80±0.31Cc
Lactic acid (1%)	4.31±0.20Aabc	4.14±0.27ABabc	3.81±0.19Bbc	2.57±0.15Cc
Lactic acid (2%)	4.12±0.18Abc	3.98±0.42Abc	2.82±0.32Bef	< 1.70Cd
Malic acid (1%)	4.47±0.23Aabc	3.64±0.51Bcd	3.47±0.60Bcde	2.78±0.07Bc
Malic acid (2%)	4.07±0.43Ac	3.63±0.34Abcd	2.68±0.50Bfg	2.18±0.36Bc
Citric acid (1%)	4.25±0.11Abc	4.09±0.36Aabc	3.47±0.37Bbcd	2.74±0.20Cc
Citric acid (2%)	4.08±0.28Abc	3.07±0.10Bd	2.18±0.15Cg	< 1.70Dd

[a] Population before treatment was 6.94 log CFU/apple.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table VI-2. Effect of organic acid treatment on inactivation of *S*. Typhimurium on whole, red, unwaxed apples.

Treatment (Organic acid)	Number of *S.* Typhimurium (log CFU/apple)[a]			
	0.5 min	1 min	5 min	10 min
DW	5.97±0.58Aa[b]	5.60±0.62Aa	5.54±0.40Aa	5.12±0.17Aa
Propionic acid (1%)	4.75±0.71Abcd	4.36±0.47Abc	3.99±0.13Ab	2.96±0.54Bb
Propionic acid (2%)	4.20±0.11Acd	3.57±0.31Bc	2.37±0.30Ccd	2.37±0.50Cb
Acetic acid (1%)	4.94±0.28Ab	4.68±0.42Ab	3.81±0.83Bbc	2.52±0.17Bb
Acetic acid (2%)	4.85±0.28Abc	4.12±0.27Abc	3.58±0.71Bbc	2.43±0.17Cb
Lactic acid (1%)	4.28±0.23Abcd	3.83±0.39Abc	3.76±0.76ABbc	2.82±0.46Bb
Lactic acid (2%)	4.33±0.16Abcd	3.64±0.08Ac	3.01±0.85Bcd	< 1.70Cd
Malic acid (1%)	4.64±0.27Abcd	4.44±0.47Abc	2.89±0.78Bcd	2.85±0.16Bb
Malic acid (2%)	4.64±0.17Abc	4.27±0.83Abc	1.92±0.17Bd	< 1.70Cd
Citric acid (1%)	4.74±0.09Abc	4.42±0.29ABbc	3.40±0.22Bbc	1.70±1.08Cc
Citric acid (2%)	3.98±0.13Ad	3.70±0.19Ac	< 1.70Be	< 1.70Bd

[a] Population before treatment was 7.37 log CFU/apple.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table VI-3. Effect of organic acid treatment on inactivation of *L. monocytogenes* on whole, red, unwaxed apples.

Treatment (Organic acid)	Number of *L. monocytogenes* (log CFU/apple)[a]			
	0.5 min	1 min	5 min	10 min
DW	5.18±0.48Aa[b]	4.67±0.43Aa	4.62±0.50Aa	4.60±0.11Aa
Propionic acid (1%)	4.40±0.34Aab	4.00±0.09ABabc	3.72±0.88BCb	2.40±0.49Cb
Propionic acid (2%)	4.28±0.09Aab	3.54±0.40Bcd	2.88±0.42Cbcd	2.10±0.31Dbc
Acetic acid (1%)	4.49±0.46Aab	4.24±0.32Aab	2.52±0.20Bbcd	2.18±0.36Bbc
Acetic acid (2%)	4.37±0.41Aab	3.52±0.17ABcd	2.60±0.24Bbcd	1.82±1.14Cbcd
Lactic acid (1%)	4.85±0.55Aab	4.29±0.29Aab	3.43±0.92Bbc	< 1.70Ce
Lactic acid (2%)	4.26±0.14Aab	3.56±0.44Bcd	2.18±0.49Ccd	< 1.70De
Malic acid (1%)	4.32±0.68Ab	4.11±0.32Aabc	1.92±0.28Bd	< 1.70Be
Malic acid (2%)	4.27±0.25Ab	3.86±0.17Abcd	2.07±0.30Bcd	<1.70Ce
Citric acid (1%)	4.19±0.39Ab	4.05±0.52Abcd	2.68±0.50Bbcd	2.00±0.35Bbc
Citric acid (2%)	3.93±0.08Ab	3.40±0.30Ad	2.12±0.32Bcd	<1.70Ce

[a] Population before treatment was 7.26 log CFU/apple.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table VI-4. Effect of organic acid treatment on inactivation of *E. coli* O157:H7 on lettuce.

Treatment (Organic acid)	Number of *E. coli* O157:H7 (log CFU/g)[a]			
	0.5 min	1 min	5 min	10 min
DW	6.71±0.48Aa[b]	6.63±0.38Aa	6.44±0.44Aa	6.09±0.43Aa
Propionic acid (1%)	6.37±0.26Aab	5.87±0.67ABab	5.15±0.91ABb	4.89±0.66Bb
Propionic acid (2%)	6.06±0.34Abc	5.70±0.55Ab	4.88±0.51Bbc	4.57±0.29Bbc
Acetic acid (1%)	6.36±0.35Aab	5.98±0.11ABab	5.19±0.55BCb	4.96±0.54Cb
Acetic acid (2%)	6.08±0.29Abc	5.82±0.21Ab	4.87±0.58Bbc	4.68±0.65Bbc
Lactic acid (1%)	6.15±0.15Abc	5.50±0.21Bb	4.40±0.26Cbcd	4.05±0.56Cbcd
Lactic acid (2%)	5.89±0.24Abc	5.25±0.55Ab	3.81±0.69Bd	3.66±0.78Bcd
Malic acid (1%)	5.86±0.31Abc	5.71±0.24Ab	4.33±0.37Bbcd	3.61±0.86Bcd
Malic acid (2%)	5.63±0.40Ac	5.41±0.46Ab	3.90±0.36Bcd	3.37±0.75Bd
Citric acid (1%)	6.13±0.10Abc	5.64±0.34Ab	4.53±0.43Bbcd	3.84±0.67Bbcd
Citric acid (2%)	5.93±0.22Abc	5.28±0.63Ab	4.41±0.27Bbcd	3.23±0.46Cd

[a] Population before treatment was 7.03 log CFU/g.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table VI-5. Effect of organic acid treatment on inactivation of *S.* Typhimurium on lettuce.

Treatment (Organic acid)	Number of *S.* Typhimurium (log CFU/g)[a]			
	0.5 min	1 min	5 min	10 min
DW	6.98±0.10Aa[b]	6.40±0.38Ba	6.19±0.19Ba	5.70±0.00Ca
Propionic acid (1%)	6.18±0.15Abc	6.05±0.12Aab	5.58±0.77ABab	4.77±0.51Bb
Propionic acid (2%)	6.02±0.18Abcd	5.80±0.09Aabc	5.07±0.56Bbcd	4.48±0.20Bbc
Acetic acid (1%)	6.20±0.36Abc	5.97±0.49ABab	5.23±0.34Bbc	4.06±0.42Cbcd
Acetic acid (2%)	5.97±0.29Acd	5.61±0.71Abc	4.50±0.69ABcde	3.96±0.42Bbcd
Lactic acid (1%)	6.30±0.30Abc	5.66±0.25Babc	4.53±0.25Ccde	3.83±0.31Dcde
Lactic acid (2%)	6.04±0.24Abcd	5.46±0.13Abc	4.26±0.79Bcde	3.16±0.25Cde
Malic acid (1%)	5.92±0.14Acd	5.48±0.07Abc	4.41±0.64CBcde	3.38±0.88Bde
Malic acid (2%)	5.79±0.54Acd	5.12±0.12Ac	3.61±0.12Be	2.72±0.66Ce
Citric acid (1%)	6.59±0.11Aab	5.51±0.63Bbc	4.39±0.61Ccde	3.85±0.56Cbcd
Citric acid (2%)	5.48±0.59Ad	5.26±0.65Abc	4.17±0.32Bde	3.38±0.29Bde

[a] Population before treatment was 7.04 log CFU/g.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table VI-6. Effect of organic acid treatment on inactivation of *L. monocytogenes* on lettuce.

Treatment (Organic acid)	Number of *L. monocytogenes* (log CFU/g)[a]			
	0.5 min	1 min	5 min	10 min
DW	6.03±0.56Aa[b]	5.92±0.57Aa	5.62±0.31Aa	5.27±0.14Aa
Propionic acid (1%)	6.02±0.25Aa	5.60±0.16Aa	5.03±0.25Bab	4.26±0.24Cb
Propionic acid (2%)	5.54±0.59Aab	5.24±0.38ABab	4.72±0.21BCbc	4.09±0.25Cbc
Acetic acid (1%)	6.01±0.69Aa	5.04±0.74Aabc	4.71±0.66Abc	3.90±0.47Abc
Acetic acid (2%)	5.33±0.65Aabc	4.34±0.59Bbcd	4.06±0.23Bcde	3.58±0.14Bcd
Lactic acid (1%)	5.57±0.38Aab	4.57±0.23Bbcd	4.03±0.49Bcde	3.06±0.32Cde
Lactic acid (2%)	4.43±0.30Ac	3.90±0.59ABd	3.49±0.51BCde	2.79±0.29Cef
Malic acid (1%)	5.13±0.11Aabc	4.40±0.70ABbcd	3.67±0.89BCbc	2.67±0.29Cef
Malic acid (2%)	4.41±0.49Ac	4.20±0.56Acd	3.12±0.39Be	2.31±0.39Bf
Citric acid (1%)	5.17±0.51Aabc	4.53±0.25Abcd	4.32±0.34Abcd	3.21±0.58Bde
Citric acid (2%)	4.83±0.43Abc	3.82±0.41Bd	3.54±0.66Bde	2.85±0.46Bef

[a] Population before treatment was 6.45 log CFU/g.

[b] Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Color analysis. No significant ($p > 0.05$) differences in Hunter's color values (L, a, b) were observed between apples treated with distilled water (control) and those with organic acids, when color of samples was analyzed (data not shown). Hunter L and b values of organic acid treated lettuce were not significantly different from those of the control treated sample (data not shown). However, differences in Hunter a values between treatments were observed during storage (Table VI-7). Hunter a value of lettuce treated with 2% acetic acid for 10 min significantly ($p < 0.05$) increased after 3 days, which means greenness of lettuce decreased. After 7 days, a significant ($p < 0.05$) increase in Hunter a value of samples treated with 2% proponic, 1% acetic, and 2% acetic acid for 10 min was observed.

Table VI-7. Hunter a value of lettuce after organic acid treatment.

Treatment Time	Storage time (days)	Organic acids[a]										
		DW	PA 1%	PA 2%	AA 1%	AA 2%	LA 1%	LA 2%	MA 1%	MA 2%	CA 1%	CA 2%
10 min	0	-14.67A[b]	-13.79A	-13.87A	-14.10A	-12.65A	-13.06A	-13.06A	-13.92A	-13.52A	-13.67A	-13.89A
	3	-12.94B	-14.15B	-13.17B	-12.93B	-9.67A	-13.70B	-12.41B	-12.95B	-13.79B	-12.65B	-14.90B
	7	-13.33B	-13.10B	-9.64A	-7.43A	-8.77A	-14.96B	-12.91B	-13.78B	-15.47B	-14.74B	-13.90B

[a] DW, Distilled water; PA, Propionic acid; AA, Acetic acid; LA, Lactic acid; MA, Malic acid; CA, Citric acid.
[b] Within the same storage time, means with different uppercase letters within a row are significantly different ($p < 0.05$).

VI-1.5. Discussion

Efficacy of organic acids to decontaminate foodborne pathogens was similar or superior to those of chlorine based sanitizers evaluated in other studies. Populations of *L. monocytogenes* on iceberg lettuce were reduced (1.7 log CFU/g) when treated with 100 ppm of chlorinated water for 5 min (Akbas and Ö lmez, 2007). Reduction in the number of *E. coli* O157:H7 on Romaine lettuce (0.65 log CFU/g) was obtained with 200 ppm chlorine dip for 2 min (Keskinen et al., 2009). Chlorine dioxide solution (200 ppm) reduced the population of *E. coli* O157:H7 on whole apples by 3 log, after 5 min dipping treatment (Wisniewsky et al., 2000).

Dramatic differences in microbial reduction were observed between control and organic acid treatment with increasing treatment time. Among tested organic acids, lactic, malic, and citric acid showed greater reduction of microbial levels than did propionic and acetic acid on both apples and lettuce. In a similar study, treatment with either 1% lactic or citric acid resulted in a significant decrease in the number of *E. coli* (3.0 log CFU/g and 3.1 log CFU/g, respectively), and *L. monocytogenes* (2.2 log CFU/g and 1.8 log CFU/g, respectively) (Akbas and Ö lmez, 2007). Lactic and citric acids were more effective than acetic acid (2.4 log reduction in *E. coli* and 1.4 log reduction in *L. monocytogenes*). Also, citric and lactic acid were more effective than acetic acid when lettuce inoculated *E. coli* O157:H7 and *L. monocytogenes* was treated with 1% organic acids for 1 min (Yuk et al., 2006).

It was noticed that the pH levels of lactic, malic, and citric acid (the most effective) were lower than the other two, propionic and acetic acid. Generally, antimicrobial action of organic acids is due to pH reduction in the environment. pH level in fact contributes to antimicrobial activity of organic acids, but many other factors, such as chain length, degree of branching, and the ratio of undissociated forms of organic acid all affect antimicrobial activity of organic acids (Doores, 1993).

In the present study, organic acids had a more pronounced effect on apples than on lettuce, although the extent of inactivation among different acids varied. Studies conducted by other researchers support the results of this study. In dip inoculated lettuce, bacterial cells were located in cut edges and damaged lettuce tissue that is less accessible to sanitizers (Seo and Frank, 1999). Also Keskinen et al. (2009) found *E. coli* O157:H7 remained in damaged lettuce tissue after 2 min treatment with 200 ppm chlorine.

The results of this study indicate that the effectiveness of organic acids as sanitizers also depends on the target pathogen. *L. monocytogenes* was relatively more sensitive to acid treatment than *E. coli* O157:H7 and *S.* Typhimurium. Similar results were observed in other investigations. Benjamin and Datta (1995) reported that enterohemorrhagic *E. coli* O157:H7, but not all strains, is acid tolerant. *E. coli* O157:H7 is relatively tolerant to organic acids (Conner and Kotrola, 1995). Also

enterobacteria are less inhibited by acid treatment than *L. monocytogenes* (Ö stling and Lindgren, 1993).

Maintenance of color after sanitizer treatment is important for consumption of fresh produce. Water rinsing after organic acid treatment used in this study may minimize color changes of apples and lettuce. Thus, organic acid treatment followed by proper water rinsing may be necessary for fresh produce processing.

In conclusion, it is suggested that organic acids have a potential application as sanitizers for organic fresh produce. Organic acids may be such a good alternative sanitizer to chemical sanitizers for sanitation of organic fresh fruits and vegetables. Also these data can assist the organic produce industry in providing safe organic fresh produce to consumers.

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VI-2. Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers

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VI-2.1. Abstract

The objective of this study was to determine the effect of aerosolized sanitizers on the inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms. Biofilms were formed on a stainless steel and polyvinyl chloride (PVC) coupon by using a mixture of three strains each of three foodborne pathogens. Six day old biofilms on stainless steel and PVC coupons were treated with aerosolized sodium hypochlorite (SHC; 100 ppm) and peracetic acid (100, 200, and 400 ppm) in a model cabinet for 5, 10, 30, and 50 min. Treatment with 100 ppm PAA was more effective than the same concentration of SHC with increasing treatment time. Exposure to 100 ppm SHC and PAA for 50 min significantly ($p < 0.05$) reduced biofilm cells of three foodborne pathogens (0.50 to 3.63 log CFU/coupon and 2.83 to more than 5.78 log CFU/coupon, respectively) compared to the control treatment. Exposure to 200 and 400 ppm PAA was more effective in reducing biofilm cells. Biofilm cells were reduced to below the detection limit (1.48 log CFU/coupon) between 10 and 30 min of exposure. The results of this study suggest that aerosolized sanitizers have a potential as a biofilm control method in the food industry.

VI-2.2. Introduction

Biofilm can be defined as a community of microbes attached to each other and embedded in an organic polymer matrix, adhering to a surface (Carpentier and Cerf, 1993; Costerton, 1995). It is a natural tendency of bacteria to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of extracellular polymeric substances that they produce, forming a biofilm (Simões et al., 2010). Biofilm exists in a variety of environments, including food processing industry (Zottola and Sasahara, 1994). Several factors in the food processing environment, such as flowing water, attachment surfaces, and sufficient nutrients, pH, water activity (a_w), and temperature are suitable for biofilm formation (Brooks and Flint, 2008; Gibson et al., 1999). Also biofilm formation of microorganisms was affected by environmental parameters, including pH, a_w, and temperature (Giaouris et al., 2005). In addition, gene expression patterns of bacteria are related to the production of biofilms. Differential gene expression compared with that of planktonic bacteria is required to form biofilms (Becker et al., 2001; Beloin and Ghigo, 2005). Several foodborne pathogens including *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* have been reported to produce biofilms (Chae and Schraft, 2000; Wirtanen et al., 2000), and the presence of biofilms can lead to contamination of food product (Hall-Stoodley et al., 2004).

It is difficult to completely remove biofilms formed on food processing surfaces

(Baumann et al., 2009). Regular disinfection is one strategy to avoid biofilm formation. However, it is hard to disinfect food processing surfaces frequently (Meyer, 2003). Also, it is hard to disinfect inaccessible surfaces such as storage tank, pump exteriors, walls, and ceilings (Chmielewski and Frank, 2003; Kumar and Anand, 1998). It may result in biofilms formation. Also pipe lines and bends in pipes are common sources related with biofilm formation (Wong, 1998).

Various cleaning methods such as electrolyzed water (Ayebah et al., 2005), ultrasound (Baumann et al., 2009), irradiation (Byun et al., 2007), and bacteriophage (Sharma et al., 2005) have been evaluated to remove biofilms on food processing surfaces. Also washing with various sanitizers including peracetic acid (Fatemi and Frank, 1999), chlorine (Joseph et al., 2001), hydrogen peroxide (DeQueiroz and Day, 2007), and ozone (Robbins et al., 2005) have been tested to control microbial biofilms. However, these methods could not be applied to clean inaccessible environmental surfaces.

Aerosolization is the dispersion of a liquid material as a fine mist in air (Oh et al., 2005b). This technique has been applied for room disinfection. Although spray sanitizing may be effective in disinfection of environmental surfaces (Gibson et al., 1999), fine aerosol mists have better penetration property than trigger spray in assessment of surface bioburden (Hiom et al., 2003). Gaseous sanitizers also could be applied in disinfection of inaccessible environmental surfaces (Vaid et al., 2010). However, sophisticated equipment is needed to generate gaseous sanitizer, and the

number of applicable sanitizers is limited (Oh et al., 2005a). In comparison, aerosolization technique have broad spectrum of applicable sanitizers.

This study was conducted to evaluate the effectiveness of aerosolized sodium hypochlorite (SHC) and peracetic acid against biofilm of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* formed on stainless steel and PVC. Different concentrations of sanitizer and exposure times were evaluated to guide appropriate application of aerosolization technique.

VI-2.3. Materials and Methods

Bacterial cultures and cell suspension. Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the School of Food Science bacterial culture collection, Washington State University (Pullman, WA, USA). Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 ˚C for 24 h, harvested by centrifugation at 4000 × *g* for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile phosphate-buffered saline (PBS; pH 7.4), corresponding to approximately 10^7-10^8 CFU/ml.

Preparation of stainless steel and PVC coupons. Stainless steel (no. 4 grade) and PVC were fabricated into coupons (5 cm × 2 cm). Stainless steel and PVC coupons were washed in detergent solution, immersed in 15% phosphoric acid solution for 20 min, and rinsed with distilled water. After washing, coupons were dried in laminar flow biosafety hood (22 ± 2 ℃) for 3 h.

Biofilm formation. Biofilm was formed by two different procedures. In the procedure A, the method used for biofilm formation was similar to that as described by Kim et al. (2006). Each prepared stainless steel and PVC coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea)

containing 30 ml of each cell suspension of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* (ca. 10^7–10^8 CFU/ml. Conical centrifuge tubes were incubated at 4 ℃ for 24 h to facilitate attachment of cell. After incubation, coupons were removed from conical centrifuge tubes with a sterile forceps, and washed in 500 ml of distilled water with gentle agitation for 5 s (22 ± 2 ℃). Washed coupons were transferred to 50-ml conical centrifuge tube containing 30 ml of TSB, and incubated at 25 ℃ for 6 days. In the procedure B, each prepared stainless steel and PVC coupon was inoculated with 0.1 ml of each cell suspension of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* (ca. 10^7–10^8 CFU/ml) by depositing droplets with a micropipet at 10 locations. After inoculation, coupons were dried in laminar flow biosafety hood for 3 h (22 ± 2 ℃) to facilitate the attachment of bacteria. Coupons were transferred 50-ml conical centrifuge tube containing 5 ml of distilled water, and incubated at 25 ℃ for 6 days.

Sanitizer preparation. Sodium hypochlorite solution (100 ppm) was prepared by adding bleach containing ≥ 4% active chlorine (Yuhan Clorox, Seoul, Korea) to distilled water. Peracetic acid (Daesung C&S, Seoul, Korea) was diluted according to the manufacturer's instruction with distilled water to 100, 200, and 400 ppm. The free chlorine concentration was measured with a HI 95771 Chlorine Ultra High Range Meter (Hanna Instruments, Ann Arbor, MI, USA).

Antibacterial aerosol treatment. Coupons were transferred to a model glass cabinet (80×50×50 cm) for aerosolized sanitizer treatment. Coupons immersed in

TSB were washed in 500 ml of distilled water with gentle agitation for 10 s (22 ± 2 ℃) before treatment. Aerosolized sanitizers generated by nebulizer (DRWL-2000, Doore Industrial Co., Gyeonggi, Korea) were put into a cabinet. Size of aerosolized particle was approximately 5.42 to 11.42 μm. Coupons were treated with aerosolized SHC (100 ppm) and PAA (100, 200, and 400 ppm) for 5, 10, 30, and 50 min. All experiments were performed at room temperature (22 ± 2 ℃).

Bacterial enumeration. After treatment, stainless steel and PVC coupons were transferred to sterile 50-ml conical centrifuge tube containing 30 ml of PBS and 3 g of glass beads (425-600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then vortexed for 1 min. Cell suspension was tenfold serially diluted in BPW, and 0.1 ml of undiluted cell suspension or diluents was spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium. The plates were incubated at 37˚C for 24–48 h. After incubation, colonies were counted.

Statistical analysis. All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC,

USA) and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

VI-2.4. Results

In the procedure A with stainless steel, initial attached cell (2-3 log) increased by 4-5log after 6days incubation (data not shown). Table VI-8 shows survival of biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after aerosolized sanitizer treatment. *E. coli* O157:H7 formed biofilm on stainless steel with a cell density of 6.44 log CFU/coupon. Biofilm cells of *E. coli* O157:H7 were significantly ($p < 0.05$) reduced by 2.56 log CFU/coupon after 50 min of exposure to 100 ppm SHC compared to distilled water treatment (control treatment). Levels of biofilm cells were reduced to below the detection limit (1.48 log CFU/coupon) after exposure to 100 ppm PAA for 50 min, and 200 and 400 ppm PAA for 10 min. The initial biofilm cells of *S.* Typhimurium were 7.09 log CFU/coupon. Treatment with 100 ppm SHC for 50 min significantly ($p < 0.05$) reduced biofilm cells by 0.50 log CFU/coupon compared to the control treatment. Treatment of biofilm cells with 100 ppm PAA brought significant reduction (4.37 log CFU/coupon) in counts compared to the control treatment after 50 min of exposure. Biofilm cells of *S.* Typhimurium could not be detected after exposure to 200 and 400 ppm PAA for 10 min. The cell density of *L. monocytogenes* biofilm was 6.18 log CFU/coupon. The biofilm cells of *L. monocytogenes* were significantly ($p < 0.05$) reduced by 2.46 and more than 4.58 log CFU/coupon, respectively, after exposure to 100 ppm SHC and PAA for 50 min

compared to the control treatment. Biofilm cells were reduced to below the detection limit (1.48 log CFU/coupon) after 30 min of exposure to 200 and 400 ppm PAA.

In the procedure B with stainless steel, bacterial populations increased by approximately 1–1.5 log CFU/coupon from initial inoculation levels of foodborne pathogens (data not shown). Biofilm cells formed by the procedure B were showed a similar reduction patterns to those formed by the procedure A (Table VI-9). Exposure to 200 and 400 ppm PAA was more effective than 100 ppm SHC in reducing biofilm cells with increasing the treatment time.

The results for inactivation of biofilm formed on PVC by aerosolized sanitizers were represented in Table VI-10 to 11. Reduction patterns of biofilm cells on PVC were similar to those of biofilm cells on stainless steel. With increasing treatment time, PAA treatment was more effective than treatment with same concentration of SHC. Also similar to biofilms on stainless steel, biofilms formed on PVC by the procedure B were more resistant to aerosolized sanitizers than these formed by the procedure A.

Table VI-8. Survival of biofilm cells of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* formed by procedure A on stainless steel coupon after aerosolized sanitizer treatment[a].

Microorganism	Treatment[b]	Population (log CFU/coupon)			
		5 min	10 min	30 min	50 min
E. coli O157:H7	DW	5.64 ± 0.49A[c]	5.48 ± 0.70A	5.42 ± 0.16A	5.27 ± 0.49A
	SHC (100 ppm)	5.22 ± 0.91A	4.76 ± 0.79A	4.00 ± 0.38C	2.71 ± 1.13B
	PAA (100 ppm)	5.40 ± 0.55A	5.25 ± 0.15A	4.96 ± 0.08B	< 1.48C
	PAA (200 ppm)	2.66 ± 1.18B	< 1.48B	< 1.48D	< 1.48C
	PAA (400 ppm)	2.48 ± 1.48B	< 1.48B	< 1.48D	< 1.48C
S. Typhimurium	DW	6.64 ± 0.26A	6.59 ± 0.49A	6.43 ± 0.25A	5.88 ± 0.08A
	SHC (100 ppm)	5.52 ± 0.82A	5.50 ± 0.15C	5.30 ± 0.26B	5.38 ± 0.22B
	PAA (100 ppm)	6.29 ± 0.21A	6.16 ± 0.07B	5.02 ± 0.28B	1.51 ± 0.05C
	PAA (200 ppm)	2.81 ± 1.28B	< 1.48D	< 1.48C	< 1.48D
	PAA (400 ppm)	2.62 ± 1.01B	< 1.48D	< 1.48C	< 1.48D
L. monocytogenes	DW	6.10 ± 0.22A	6.01 ± 0.31A	6.07±0.31A	6.06 ± 0.02A
	SHC (100 ppm)	4.98 ± 0.90A	4.58 ± 0.73B	3.75±0.07B	3.60 ± 0.98B
	PAA (100 ppm)	5.60 ± 0.20A	5.35 ± 0.46AB	3.79±0.07B	< 1.48C
	PAA (200 ppm)	3.27 ± 0.79B	1.98 ± 0.50C	< 1.48C	< 1.48C
	PAA (400 ppm)	2.18 ± 0.70C	1.81 ± 0.58C	< 1.48C	< 1.48C

[a] Mean ± standard deviation; n = 3 for all treatments.
[b] DW, Distilled water; SHC, Sodium hypochlorite; PAA, Peracetic acid.
[c] Values in the same row that are not followed by the same capital letters are significantly different ($p < 0.05$).

Table VI-9. Survival of biofilm cells of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* formed by procedure B on stainless steel coupon after aerosolized sanitizer treatment[a].

Microorganism	Treatment[b]	Population (log CFU/coupon)			
		5 min	10 min	30 min	50 min
E. coli O157:H7	DW	7.70 ± 0.52A[c]	7.71 ± 0.42A	7.75 ± 0.35A	7.72 ± 0.40A
	SHC (100 ppm)	7.54 ± 0.37A	7.58 ± 0.39A	7.40 ± 0.73A	5.76 ± 0.49B
	PAA (100 ppm)	7.62 ± 1.16A	7.27 ± 0.03A	5.58 ± 0.43B	4.20 ± 0.12C
	PAA (200 ppm)	6.75 ± 0.29A	5.73 ± 0.24B	< 1.48C	< 1.48D
	PAA (400 ppm)	6.59 ± 0.25A	5.33 ± 0.03B	< 1.48C	< 1.48D
S. Typhimurium	DW	8.06 ± 0.14A	8.05 ± 0.10A	7.86 ± 0.44A	7.80 ± 0.11A
	SHC (100 ppm)	7.83 ± 0.03 A	7.52 ± 0.12B	7.37 ± 0.56A	6.07 ± 0.71B
	PAA (100 ppm)	7.23 ± 0.85AB	7.14 ± 0.08C	5.09 ± 0.05B	2.02 ± 0.78C
	PAA (200 ppm)	6.85 ± 0.38B	5.98 ± 0.04D	< 1.48C	< 1.48D
	PAA (400 ppm)	6.71 ± 0.35B	5.90 ± 0.10D	< 1.48C	< 1.48D
L. monocytogenes	DW	7.04 ± 0.07A	7.11 ± 0.73A	7.02 ± 0.94A	6.68 ± 0.80A
	SHC (100 ppm)	6.91 ± 0.39AB	6.37 ± 0.67A	5.79 ± 0.52AB	4.30 ± 0.71B
	PAA (100 ppm)	5.91 ± 1.09B	5.30 ± 0.61B	5.05 ± 1.16B	3.64 ± 0.10B
	PAA (200 ppm)	6.00 ± 0.42AB	5.17 ± 0.17B	< 1.48C	< 1.48C
	PAA (400 ppm)	3.84 ± 0.07C	1.98 ± 0.50C	< 1.48C	< 1.48C

[a] Mean ± standard deviation; n = 3 for all treatments.
[b] DW, Distilled water; SHC, Sodium hypochlorite; PAA, Peracetic acid.
[c] Values in the same row that are not followed by the same capital letters are significantly different ($p < 0.05$).

Table VI-10. Survival of biofilm cells of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* formed by procedure A on PVC coupon after aerosolized sanitizer treatment[a].

Microorganism	Treatment[b]	Population (log CFU/coupon)			
		5 min	10 min	30 min	50 min
E. coli O157:H7	DW	6.18 ± 0.96A[c]	5.96 ± 0.41A	5.87 ± 0.49A	5.88 ± 0.38A
	SHC (100 ppm)	5.19 ± 1.05AB	4.69 ± 0.14C	4.06 ± 0.74B	3.54 ± 0.22B
	PAA (100 ppm)	5.66 ± 0.52AB	5.45 ± 0.19B	3.43 ± 0.22B	1.57 ± 0.17C
	PAA (200 ppm)	4.29 ± 1.27B	< 1.48D	< 1.48C	< 1.48D
	PAA (400 ppm)	1.49 ± 0.10C	< 1.48D	< 1.48C	< 1.48D
S. Typhimurium	DW	6.74 ± 0.36A	6.79 ± 0.45A	6.75 ± 0.58A	6.39 ± 0.59A
	SHC (100 ppm)	6.20 ± 0.10B	4.79 ± 0.60C	4.06 ± 0.24B	2.76 ± 0.91B
	PAA (100 ppm)	5.73 ± 0.65BC	5.73 ± 0.15B	4.53 ± 0.73B	< 1.48C
	PAA (200 ppm)	5.26 ± 0.24C	< 1.48D	< 1.48C	< 1.48C
	PAA (400 ppm)	1.77 ± 0.29D	< 1.48D	< 1.48C	< 1.48C
L. monocytogenes	DW	6.44 ± 0.04A	6.40 ± 0.01A	5.75 ± 0.45A	5.29 ± 0.03A
	SHC (100 ppm)	4.68 ± 1.09BC	4.58 ± 0.29C	3.28 ± 0.44C	2.24 ± 0.68B
	PAA (100 ppm)	5.82 ± 0.44AB	5.34 ± 0.15B	4.58 ± 0.24B	< 1.48C
	PAA (200 ppm)	3.76 ± 0.92CD	< 1.48D	< 1.48D	< 1.48C
	PAA (400 ppm)	2.72 ± 1.30D	< 1.48D	< 1.48D	< 1.48C

[a] Mean ± standard deviation; n = 3 for all treatments.
[b] DW, Distilled water; SHC, Sodium hypochlorite; PAA, Peracetic acid.
[c] Values in the same row that are not followed by the same capital letters are significantly different ($p < 0.05$).

Table VI-11. Survival of biofilm cells of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* formed by procedure B on PVC coupon after aerosolized sanitizer treatment[a].

Microorganism	Treatment[b]	Population (log CFU/coupon)			
		5 min	10 min	30 min	50 min
E. coli O157:H7	DW	7.46 ± 0.26A[c]	7.49 ± 0.27A	7.39 ± 0.25A	7.01 ± 0.71A
	SHC (100 ppm)	7.27 ± 0.58AB	7.17 ± 0.07AB	6.96 ± 0.08B	5.62 ± 1.16B
	PAA (100 ppm)	7.37 ± 0.23A	6.85 ± 0.12B	5.32 ± 0.19C	4.18 ± 0.15C
	PAA (200 ppm)	6.76 ± 0.09BC	6.23 ± 0.15C	< 1.48D	< 1.48D
	PAA (400 ppm)	6.40 ± 0.09C	5.18 ± 0.45D	< 1.48D	< 1.48D
S. Typhimurium	DW	8.03 ± 0.23A	7.89 ± 0.11A	7.83 ± 0.07A	7.83 ± 0.36A
	SHC (100 ppm)	8.01 ± 0.29A	7.61 ± 0.37A	7.14 ± 0.11B	6.40 ± 1.06B
	PAA (100 ppm)	7.56 ± 0.30AB	7.42 ± 0.73AB	3.91 ± 0.13C	3.50 ± 0.15C
	PAA (200 ppm)	6.99 ± 0.58B	6.56 ± 0.61B	< 1.48D	< 1.48D
	PAA (400 ppm)	7.08 ± 0.08B	5.43 ± 0.64C	< 1.48D	< 1.48D
L. monocytogenes	DW	7.29 ± 0.70A	7.08 ± 0.64A	6.58 ± 0.20A	6.68 ± 0.95A
	SHC (100 ppm)	7.32 ± 0.51A	7.06 ± 0.75A	5.19 ± 0.24B	4.16 ± 0.32B
	PAA (100 ppm)	6.93 ± 0.20AB	6.44 ± 0.05AB	4.37 ± 0.11C	1.85 ± 0.68C
	PAA (200 ppm)	6.28 ± 0.70B	5.59 ± 0.94B	< 1.48D	< 1.48D
	PAA (400 ppm)	4.75 ± 0.05C	4.19 ± 0.06C	< 1.48D	< 1.48D

[a] Mean ± standard deviation; n = 3 for all treatments.
[b] DW, Distilled water; SHC, Sodium hypochlorite; PAA, Peracetic acid.
[c] Values in the same row that are not followed by the same capital letters are significantly different ($p < 0.05$).

VI-2.5. Discussion

Biofilms are more resistant to antimicrobial agents compared to planktonic cells, and this makes their elimination from food processing environment a big challenge (Simões and Vieira, 2009). Resistance to antimicrobial agents may be in part by virtue of complex structure or the polysaccharides (Meyer, 2003; Sharma and Anand, 2002). Thus mature biofilms rather than planctonic cells or early-stage biofilms must be considered when disinfection protocols have to be optimized (Ibusquiza et al., 2011). Several disinfection methods have been evaluated to remove biofilms on food processing surfaces. Ayebah et al. (2005) used electrolyzed water (EO) to inactivate *L. monocytogenes* biofilms on stainless steel surfaces. Treatment with acidic EO water for 30 to 120 s reduced the bacteria population by 4.3 to 5.2 log CFU/coupon. Ultrasound treatment (20 KHz, 120 W) at a distance of 2.54 cm from biofilm chip reduced levels of *L. monocytogenes* biofilms by 3.8 log CFU/ml after 60 s (Baumann et al., 2009). Recently, Chorianopoulos et al. (2011) reported nanostructured TiO_2 thin films on stainless steel and glass is alternative means of disinfecting contaminated surface. The biofilm of *L. monocytogenes* on glass decreased by 3 log CFU/cm^2 when TiO_2 was activated by ultraviolet A light for 90 min. However, effective methods to control biofilms on inaccessible area are needed in the food industry.

In the present study, aerosolized sanitizers showed significant antimicrobial effect against biofilm cells of foodborne pathogens on stainless steel and PVC. Oh et al. (2005a) reported aerosolized peroxyacetic acid exhibited a 3-4 log reduction in population of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on lettuce leaves. Gibson et al. (1999) reported high pressure (17.2, 34.5, 51.7, and 68.9 bar) spraying method with a sanitizer is effective at reducing biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, aerosol generated by high pressure spraying method should be applied in a short distance to bacterial biofilms (125–250 mm). On the other hand, aerosolization method can deliver sanitizers to inaccessible areas and have higher penetrating activity. Oh et al. (2005b) found aerosolized peroxyacetic acid and hydrogen peroxide diffused effectively in huge semi-trailer. Levels of *Bacillus cereus*, *Listeria innocua*, *S. aureus*, and *S.* Typhimurium were reduced by an average of 3.09, 7.69, 6.93, and 8.18 log units per plate, respectively. Also they reported aerosolized sanitizers represented antimicrobial effect regardless of height and orientation. These properties may facilitate the use of aerosolization method as a sanitation procedure of food processing plants. It may effectively reduce biofilm cells of foodborne pathogens in inaccessible food processing surfaces.

Results from this study show that aerosolized 100 ppm SHC and PAA showed a similar degree of antimicrobial effect against biofilm cells formed on stainless steel and PVC. However, with increasing treatment time, 100 ppm PAA was more

effective than 100 ppm SHC. Aerosolized PAA (200 and 400 ppm) were more effective to remove biofilms of three foodborne pathogens. Oxidizing agents including chlorine and PAA are frequently used to kill or remove biofilms (Meyer, 2003). Chlorine is known to remove exopolysaccharide as well as to kill microorganisms (Ronner and Wong, 1993). PAA widely used as peracid sanitizer, is a more potent biocide than hydrogen peroxide (Chmielewski and Frank, 2003). Several researches have been conducted to evaluate antimicrobial effect of chlorine and PAA against biofilm cells. Generally, PAA is considered to be more effective than chlorine. It maintains activity in the presence of organic loads (McDonnell and Russell, 1999). Norwood and Gilmour (2000) found that 200 ppm free chlorine treatment did not significantly reduce biofilm cells of *L. monocytogenes*. PAA sanitizer was more effective than same concentration of chlorine sanitizer for inactivation of *L. monocytogenes* and *Pseudomonas* biofilm cells grown in milk (Fatemi and Frank, 1999). Harkonen et al. (1999) reported that peroxide based sanitizers were more effective than hypochlorite for inactivation of bacterial biofilms.

Method of biofilm formation similar to the procedure A in this study has been used in other studies (Fatemi and Frank, 1999; Kim et al., 2008). In the present study, different method (procedure B) was used to form biofilms. In the procedure B, biofilms were formed by drying the bacterial suspension on stainless steel and PVC coupons. Biofilms formed by the procedure B were more resistant to aerosolized

sanitizers than these formed by the procedure A. Further study is needed to verify the high resistance of biofilm formed by the procedure B.

In conclusion, control and removal of biofilms are important issue for food processing industry. This study showed that aerosolized sanitizers were able to inactivate foodborne pathogens within biofilms effectively. It may provide cleaning procedure for food industry to control biofilms in food processing facilities.

VI-2.6. References

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VI-3. Fate of biofilm cells of *Cronobacter sakazakii* under modified atmosphere conditions

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VI-3.1. Abstract

Survival of biofilm cells of *Cronobacter sakazakii* formed on stainless steel and polyvinyl chloride (PVC) on exposure to different atmosphere conditions was studied. Biofilms were formed on stainless steel and PVC coupons by using three strains of *C. sakazakii*. Six day old biofilms on stainless steel and PVC coupons were stored under N_2 gas, CO_2 gas, and air for up to 20 days. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 1.79 and 2.47 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage. N_2 and CO_2 gases led to less reduction of biofilm cells on PVC compared to those on stainless steel. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 0.98 and 1.20 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage.

VI-3.2. Introduction

Cronobacter sakazakii is a group of Gram-negative, motile, and facultative anaerobic bacteria (Healy et al., 2010). *C. sakazakii* is a bacterium considered to be an emerging foodborne pathogen, causing a rare but severe disease in neonates (< 28 days) and children under 4 years of age which may present as septicemia, meningitis, or necrotizing enterocolitis (Adekunte et al., 2010; Hartmann et al., 2010; Iversen and Forsythe, 2003). *Cronobacter* spp. can be isolated from a range of foods such as milk, cheese, dried foods, meats, vegetables, rice, bread, tea, herbs, spices, and powdered infant formula (PIF), and PIF is considered the main source of this pathogen (Bowen & Braden, 2006; Healy et al., 2010; Iversen et al., 2004). Also *C. sakazakii* has been isolated from a variety of abiotic surfaces, and spoon and blender used in preparation of PIF are the source of infection (Chenu and Cox, 2009; Iversen and Forsythe, 2003; Kandhai et al., 2004). Contaminated PIF preparation equipment, such as spoons, blenders, and brushes have been associated with neonatal infections caused by *C. sakazakii* (Bar-Oz et al., 2001; Simmons et al., 1989).

Some strains of *C. sakazakii* have been known to produce biofilms on several materials such as stainless steel, polyvinyl chloride (PVC), latex, silicon, polycarbonate, and glass (Iversen et al., 2004; Kim et al., 2006; Lehner et al., 2005). Biofilms provide a physical barrier and protect bacterial cells against various environmental stresses such as antibiotics, sanitizers, osmotic stress, heat, and

starvation (Borucki et al., 2003; Costertonet al., 1995; Folsom and Frank, 2006; O'Tolle et al., 2000). Also, biofilms on the abiotic surfaces could be a source of contamination (Furukawa et al., 2006; Kim et al., 2008).

The survival characteristics of biofilm cells of *C. sakazakii* as affected by temperature, relative humidity (RH), or disinfectants have been studied to develop effective eliminating strategies (Kim et al., 2008; Kim et al., 2007). However, there have been no studies about the effect of modified atmosphere conditions on the survival of biofilm cells of *C. sakazakii*. Modified atmosphere is an effective food preservation method for various foods and inhibits the growth of microorganisms (Latou et al., 2014; Lu, 2009; Sivertsvik et al., 2002). This study aimed at determining survival characteristics of biofilm cells of *C. sakazakii* as affected by different atmosphere conditions. Survival of cells in biofilms formed on stainless steel and PVC on exposure to air, N_2 gas, and CO_2 gas for up to 20 days was determined.

VI-3.3. Materials and Methods

Bacterial cultures and cell suspension. Three strains of *C. sakazakii* (ATCC 12868, ATCC 29004, and FSM 30) were obtained from the School of Food Science bacterial culture collection, Washington State University (Pullman, WA, USA). These strains were cultured in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 ℃ for 24 h, harvested by centrifugation at 4000 × *g* for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile phosphate-buffered saline (PBS; pH 7.4), corresponding to approximately 10^7-10^8 CFU/ml.

Biofilm formation. The method used for biofilm formation was similar to that described by Kim, Ryu, and Beuchat (2007). Stainless steel (thickness, 0.01 cm), type 304 with number 4 finished, and PVC (thickness, 0.02 cm) were purchased from Kahee metal (Incheon, Korea) and cut into coupons (5 cm × 2 cm). Each prepared coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing 30 ml of cell suspension of *C. sakazakii* in PBS (ca. 10^7-10^8 CFU/ml). Conical centrifuge tubes were incubated at 4 ℃ for 24 h to facilitate attachment of cells. After incubation, coupons were removed from conical centrifuge tubes with a sterile forceps, and washed in 500 ml of sterile distilled water for 10 sec (22 ± 2 ℃). Washed coupons were transferred to 50-ml conical centrifuge tubes containing 30 ml of TSB, and incubated at 25 ℃ for 6 days.

Survival of biofilm cells of *C. sakazakii* as affected by different atmosphere conditions. Nylon-polyethylene vacuum bags (16 cm × 25 cm) with a film thickness of 0.07 mm manufactured by Wonchang vinyl packaging (Seoul, South Korea) were used in this study. Each stainless steel and PVC coupon was transferred to nylon-polyethylene vacuum bags separately, and packed under different atmosphere conditions (N_2 gas 100%, CO_2 gas 100 %) with a vacuum packaging machine (Airzero, Ansan, Korea). N_2 and CO_2 gas were purchased from KumKang gas (Bucheon-si, South Korea). Also, stainless steel and PVC coupons were air (control) packaged. The samples were stored at 25℃ (RH, 20 ± 2%) for 20 days and analyzed before packaging (0 day) and on 5th, 10th, 15th and 20th day of storage. Eight samples for each packaging condition were prepared and two samples were randomly taken at each storage time (5, 10, 15, and 20 days). Gas contents in the packages were measured immediately after packaging and before microbial analysis at each sampling day using a gas analyzer (OXYBABY, WITT-Gasetechnik, Germany) to confirm the leakage of gas. At each sampling day, biofilm cells were removed from stainless steel and PVC coupons by swabbing with a sterile cotton swab moistened with BPW (Kim and Wei, 2007; Ibusquiza et al., 2012). The swab was transferred to a tube containing 10 ml of BPW and vortex-mixed for 2 min to suspend the cells. Cell suspension was ten-fold serially diluted in BPW, and 0.1 ml of undiluted cell suspension or diluents was spread-plated onto HiCrome Ent.

sakazakii Agar (HiMedia, Bombay, India). The plates were incubated at 37˚C for 24 h, and colonies were counted.

Statistical analysis. All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC, USA) and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

VI-3.4. Results

Fig. VI-1 shows the survival of biofilm cells of *C. sakazakii* on stainless steel stored under different atmosphere conditions. *C. sakazakii* formed biofilms on stainless steel coupons with a cell density of 7.67 log CFU/cm^2. There were significant ($p < 0.05$) differences on levels of biofilm cells of *C. sakazakii* among samples stored under different atmosphere conditions. N_2 gas resulted in significant ($p < 0.05$) further reductions of 0.98, 1.34, 1.96, and 1.79 log CFU/cm^2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. CO_2 gas exhibited significant ($p < 0.05$) further reductions of 1.31, 1.45, 2.04, and 2.47 log CFU/cm^2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. N_2 and CO_2 gases led to less reduction of biofilm cells on PVC compared to those on stainless steel (Fig. VI-2). The initial biofilm cells of *C. sakazakii* on PVC were 7.09 log CFU/cm^2. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 0.98 and 1.20 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage.

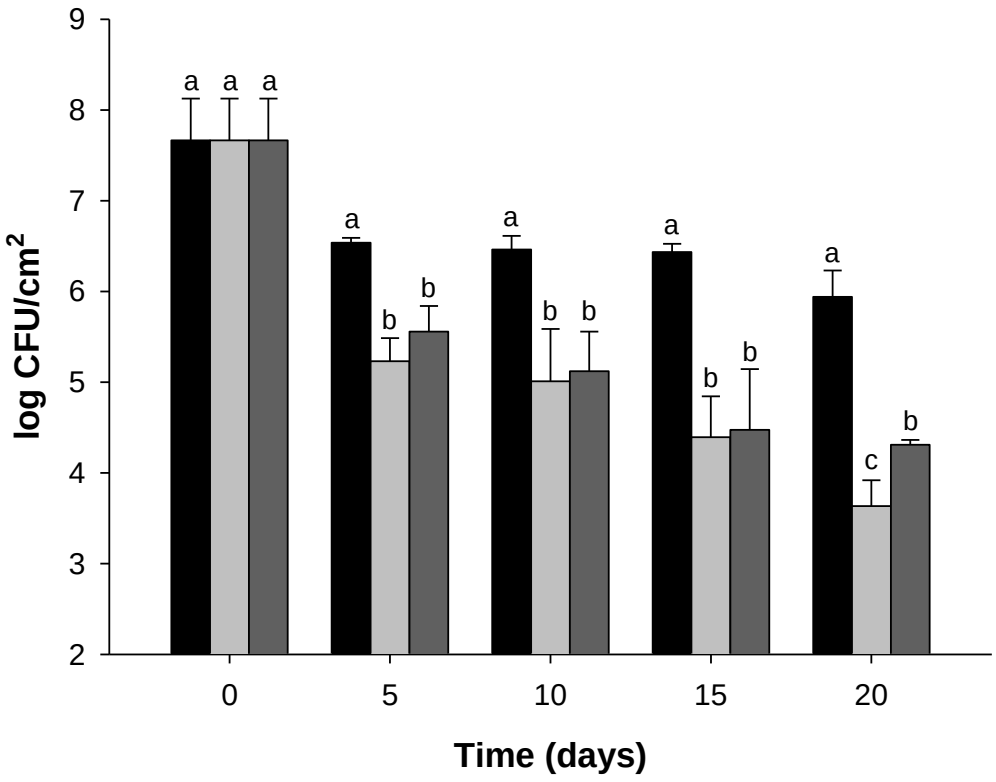

Fig. VI-1. Survival of biofilm cells of *C. sakazakii* on stainless steel coupons stored under different atmosphere conditions for up to 20 days. ■, air; ■, N_2 gas; ■, CO_2 gas. Means with the same letter within each treatment level are not significantly different ($p < 0.05$).

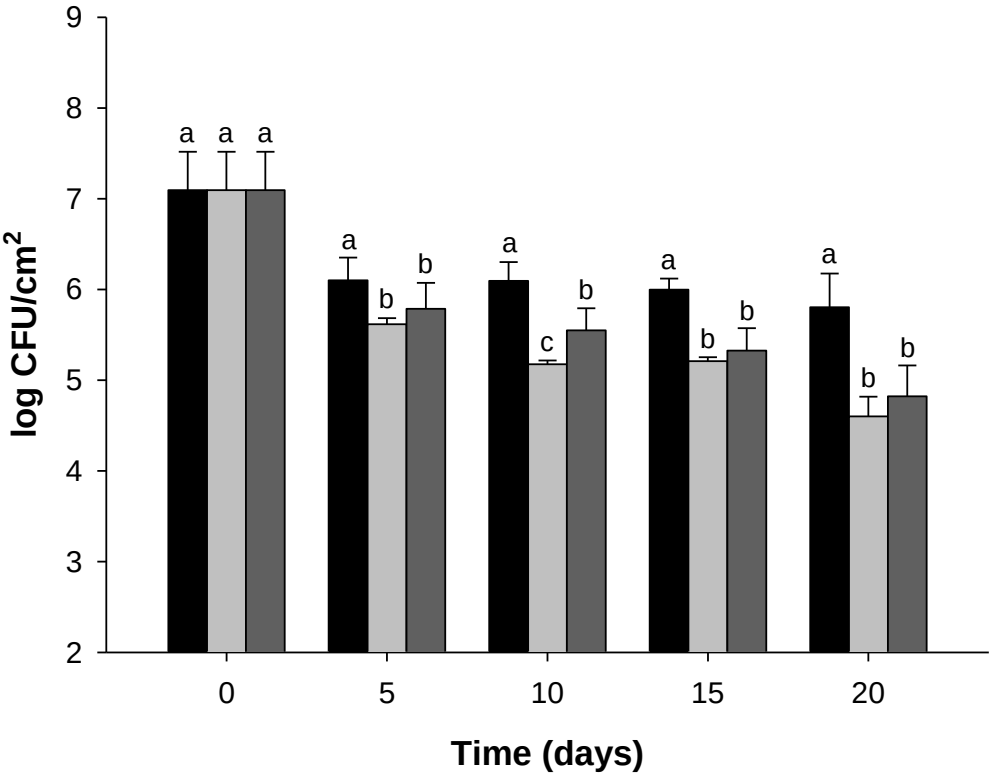

Fig. VI-2. Survival curves of biofilm cells of *C. sakazakii* on PVC coupons stored under different atmosphere conditions for up to 20 days. ■, air; ■, N_2 gas; ■, CO_2 gas. Means with the same letter within each treatment level are not significantly different ($p < 0.05$).

VI-3.5. Discussion

Kim et al. (2008) reported the numbers of biofilm cells of *C. sakazakii* on stainless steel coupon decreased significantly within 5 days at RH 23%, remained constant until 35 days of storage. Similarly, significant reduction of air packaged biofilm cells of *C. sakazakii* was observed within 5 days at RH 20%, in this study. CO_2 gas is widely used due to its antimicrobial effect. It could affect the cell membrane function, enzymatic reactions, intracellular pH, or physiochemical properties of proteins of bacteria (Davies, 1995; Farber, 1991). N_2 gas is commonly used as filler gas to prevent collapse of packaging (Mullan and McDowell, 2003). Although it could inhibit the growth of aerobic microorganisms, little is known about the antimicrobial effect of N_2 gas (Cutter, 2002). In this study, N_2 gas along with CO_2 gas significantly reduced biofilm cells of *C. sakazakii* compared to air storage. The treatment of radish seeds with ClO_2 (100 μL/mL) followed by storage under modified atmosphere (10% O_2, 10% CO_2 and 80% N_2; 5% O_2, 10% CO_2 and 85% N_2; or 10% O_2, 0% CO_2 and 90% N_2) for 1 day significantly reduced the number of *Cronobacter* spp. compared to air storage (Kim et al., 2013). Further study is needed to determine the mode of action of CO_2 and N_2 gases on survivor of biofilm cells of *C. sakazakii*.

Biofilm cells of *C. sakazakii* showed greater resistance against N_2 and CO_2 gases on PVC coupons than stainless steel coupons. Characteristics of the substratum may

affect the formation of the matrix material and the structure of the biofilms (Bremer et al., 2002; Krysinski et al., 1992). Bremer et al. (2002) reported that biofilm cells of *Listeria monocytogenes* on PVC/polyester surfaces were more resistant to chlorine treatment than those on stainless steel surfaces. The resistance of biofilm cells of *L. monocytogenes* on stainless steel was lower than that on polyester or polyester/polyurethane (Krysinski et al., 1992).

In conclusion, reductions of biofilm cells of *C. sakazakii* were greater under N_2 and CO_2 gases conditions rather than air condition. These results provide insights to predicting fate of biofilm cells of *C. sakazakii* on abiotic surfaces under modified atmosphere conditions. In this study, cocktails of *C. sakazakii* strains were used. Further studies are required to compare survival patterns of *C. sakazakii* strains with different ability in biofilm formation.

VI-3.6. References

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VI-4. Inactivation of biofilm cells of foodborne pathogens by steam pasteurization

(Published in European Food Research and Technology, 2014)

VI-4.1. Abstract

The objective of this study was to evaluate the effect of steam pasteurization on the inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms on stainless steel and polyvinyl chloride (PVC). Biofilms were formed on a stainless steel and PVC coupon by using a mixture of three strains each of three foodborne pathogens. Six day old biofilms on stainless steel and PVC coupons were treated with steam at 75 and 85 ℃ for 5, 10, 20, 30, 40 and 50 s. Biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on stainless steel were reduced by more than 6 log CFU/coupon after exposure to steam at 75 ℃ for 30, 40, and 30 s, respectively, and at 85 ℃ for 30, 20, and 20 s, respectively. Steam treatment resulted in less reduction in the levels of biofilm cells on PVC coupons. Biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were reduced by 1.78, 2.04, and 1.29 log CFU/coupon, respectively, after 50 s of exposure to steam at 75 ℃. Exposure to steam at 85° for 50 s reduced biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* by 2.53, 3.01, and 1.70 log CFU/coupon, respectively. The results of this study suggest that steam pasteurization has potential as a biofilm control method by the food industry.

VI-4.2. Introduction

Escherichia coli O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are the most relevant foodborne pathogens (Germini et al., 2009). These foodborne pathogens have been known to form biofilms on food processing surfaces such as stainless steel, plastic, polycarbonate, glass, and polyvinyl chloride (PVC) (Blackman and Frank, 1996; Borucki et al., 2003; Dewanti and Wong, 1995; Dhir and Dodd, 1995; Ryu et al., 2004). Biofilms can be defined as communities of microorganisms attached to each other and embedded in an organic polymer matrix, adhering to a surface (Carpentier and Cerf, 1993; Costerton, 1995) In the food processing environment, several factors such as flowing water, suitable attachment surfaces, pH, and sufficient nutrients favor biofilm formation (Gibson, 1999; Brooks and Flint, 2008). The attachment of bacteria and subsequent development of biofilms on food processing surfaces may cause food spoilage or transmission of diseases (Dewanti and Wong, 1995). Biofilm cells of microorganisms appear to be more resistant to heat and antimicrobial agents (Carpentier and Cerf, 1993; Costerton et al., 1999; Vaid et al., 2010; Zottola and Sasahara, 1994).

Washing with various sanitizers including peracetic acid (Fatemi and Frank, 1999), chlorine (Joseph et al., 2001), hydrogen peroxide (DeQueiroz and Day, 2007), ethylene diamino tetracetic acid (EDTA) (Chang et al., 2002), quaternary ammonium chloride (Wang et al., 2012), and ozone (Robbins et al., 2005) have been

tested as control measures for microbial biofilms. Also, various cleaning methods such as electrolyzed water (Ayebah et al., 2005), ultrasound (Baumann et al., 2009), and bacteriophages (Sharma et al., 2005) have been evaluated as ways to remove biofilms from food processing surfaces. However, these methods cannot be utilized for cleaning inaccessible areas. It is difficult to clean inaccessible environmental surfaces such as walls and ceilings, whereas product contact surfaces may be easily cleaned (Gibson et al., 1999).

Steam can increase food surface temperatures rapidly. It can transfer large amounts of heat to foods (James et al., 2000). Steam at 100 ℃ has a greater heat capacity than the same amount of water at the same temperature. Also, steam can penetrate cavities and crevices effectively (Morgan et al., 1996). Steam pasteurization for the decontamination of meat products is used extensively in many countries (Sheridan, 2004). The existing data suggests that the application of steam effectively reduces the presence of a number of pathogens such as *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*, and *Campylobacter* (McCann et al., 2006; Phebus et al., 1997; Whyte et al., 2003).

The main objective of this study was to evaluate the efficacy of steam pasteurization against biofilms of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* formed on stainless steel and PVC. Different exposure times and treatment temperatures were evaluated to guide appropriate steam pasteurization protocols for biofilms of foodborne pathogens.

VI-4.3. Materials and Methods

Bacterial cultures and cell suspension. Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19586, ATCC 43174, DT 104), and *L. monocytogenes* (ATCC 7644, ATCC 19114, ATCC 19115) were obtained from the Food Hygiene Laboratory Bacterial Culture Collection at Seoul National University (Seoul, Korea) and used in this experiment. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37˚C for 24 h, harvested by centrifugation at 4000 × g for 20 min at 4 ℃, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile phosphate-buffered saline (PBS; pH 7.4), corresponding to approximately 10^7-10^8 CFU ml^{-1}.

Preparation of stainless steel and PVC coupons and biofilm formation. Stainless steel (no. 4 grade) and PVC were fabricated into coupons (5 cm × 2 cm). Stainless steel and PVC coupons were immersed in 70% ethanol for 20 min, and rinsed with sterilized distilled water. After washing, coupons were dried in a laminar flow biosafety hood (22 ± 2 ℃) for 3 h. The method used for biofilm formation was similar to that as described by Kim et al. (2006). Each prepared stainless steel and PVC coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing 30 ml of each cell suspension of *E. coli*

O157:H7, *S.* Typhimurium, and *L. monocytogenes* in PBS (ca. 10^7-10^8 CFU/ml). Conical centrifuge tubes were incubated at 4 ℃ for 24 h to facilitate attachment of cells. After incubation, coupons were removed from conical centrifuge tubes with a sterile forceps, and washed in 500 ml of sterile distilled water for 10 sec (22 ± 2 ℃). Washed coupons were transferred to 50-ml conical centrifuge tubes containing 30 ml of TSB, and incubated at 25 ℃ for 6 days.

Steam pasteurization. A steam pasteurization apparatus developed and constructed at Seoul National University (Seoul, Korea) and Young Jin Engineering (Daegu, Korea) was used in this study. Coupons were removed from TSB and washed in 500 ml of distilled water for 10 sec (22 ± 2 ℃), and coupons were steam pasteurized at 75 ℃ and 85 ℃ for 5, 10, 20, 30, 40, and 50 s. All experiments were performed at room temperature (22 ± 2 ℃).

Bacterial enumeration. After steam treatment, stainless steel and PVC coupons were transferred to sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of glass beads (425-600 μm; Sigma-Aldrich, St. Louis, MO, USA), and then vortexed for 1 min. Cell suspension was tenfold serially diluted in 9 mL of BPW, and 0.1 ml of undiluted cell suspension or diluents was spread-plated onto the appropriate medium. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Modified Oxford Medium (MOX; Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. The plates were incubated at

37˚C for 24-48 h. For the enumeration of injured *E. coli* O157:H7, phenol red agar base with 1% sorbitol (SPRAB; Difco) was used. One hundred microliters of undiluted cell suspension or diluents were spread-plated and incubated at 37 ℃ for 24 h. Injured cells of *S.* typhimurium and *L. monocytogenes* were enumerated using the overlay (OV) method proposed by Kang and Fung [30, 31]. One hundred microliters of undiluted cell suspension or diluents were spread-plated onto TSA and incubated at 37 ℃ for 2 h to allow injured cells to resuscitate before overlaying with 7 mL of XLD (OV-XLD) or MOX (OV-MOX) for *S.* Typhimurium and *L. monocytogenes*, respectively. The plates were incubated at 37 ℃ for 22 h after the overlay solidified. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium.

Statistical analysis. All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC, USA) and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

VI-4.4. Results

Inactivation of biofilm cells formed on stainless steel. The effects of steam treatment against the biofilm cells of three tested pathogens on stainless steel are shown in Fig. VI-3, 4, and 5. The initial biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were 8.16, 7.93, and 7.54 log CFU/coupon, respectively. Steam treatment effectively reduced biofilm cells, and high reductions were achieved as steam treatment time and temperature increased. The number of biofilm cells of *E. coli* O157:H7 and *L. monocytogenes* was reduced to below the detection limit (1.48 log CFU/coupon) within 30 s when treated with steam at 75 ℃. Levels of biofilm cells of *S.* Typhimurium were reduced to below the detection limit (1.48 log CFU/coupon) after 40 s of steam treatment at 75 ℃. Significant ($p < 0.05$) further reductions were observed with steam treatment at 85 ℃. Treatment with steam at 85 ℃ for 20 s reduced the number of biofilm cells of *S.* Typhimurium and *L. monocytogenes* to below the detection limit (1.48 log CFU/coupon). Overall, the levels of injured cells enumerated on SPRAB, OV-XLD, and OV-OAB were not different from the levels of healthy cells enumerated on SMAC, XLD, and OAB. However, significant ($p < 0.05$) differences between the levels of injured cells and healthy cells were observed as steam treatment time increased.

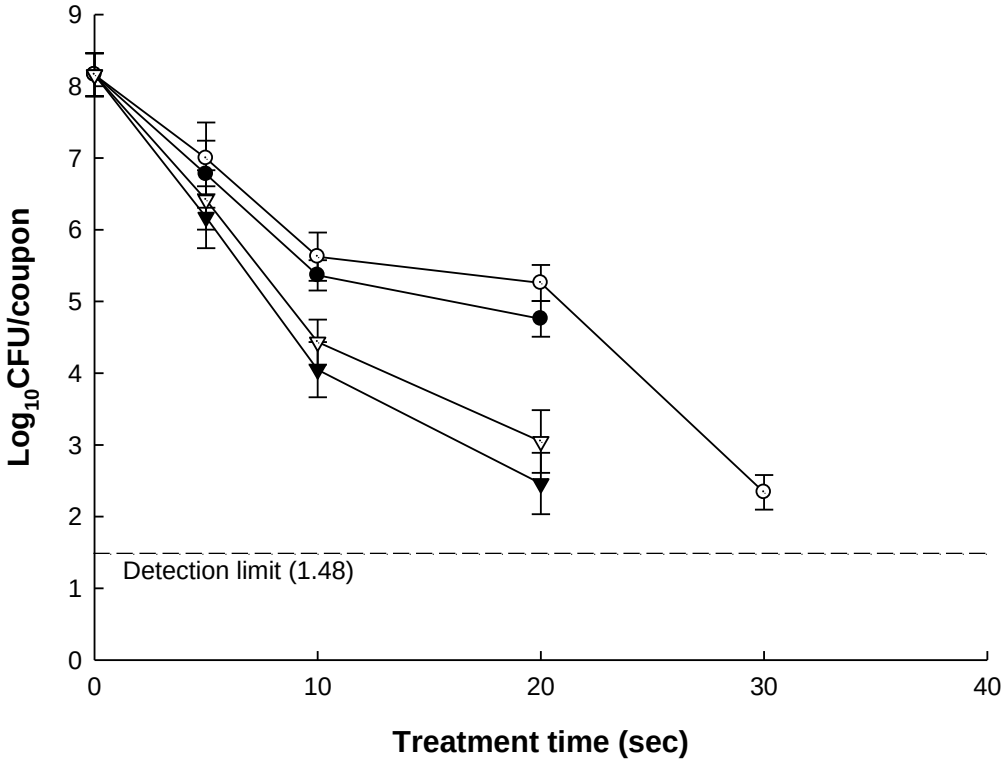

Fig. VI-3. Survival (log CFU/coupon) of biofilm cells of *Escherichia coli* O157:H7 formed on stainless steel coupons after steam pasteurization. ○, SPRAB (75 ℃); ●, SMAC (75 ℃); ▽, SPRAB (85 ℃); ▼, SMAC (85 ℃).

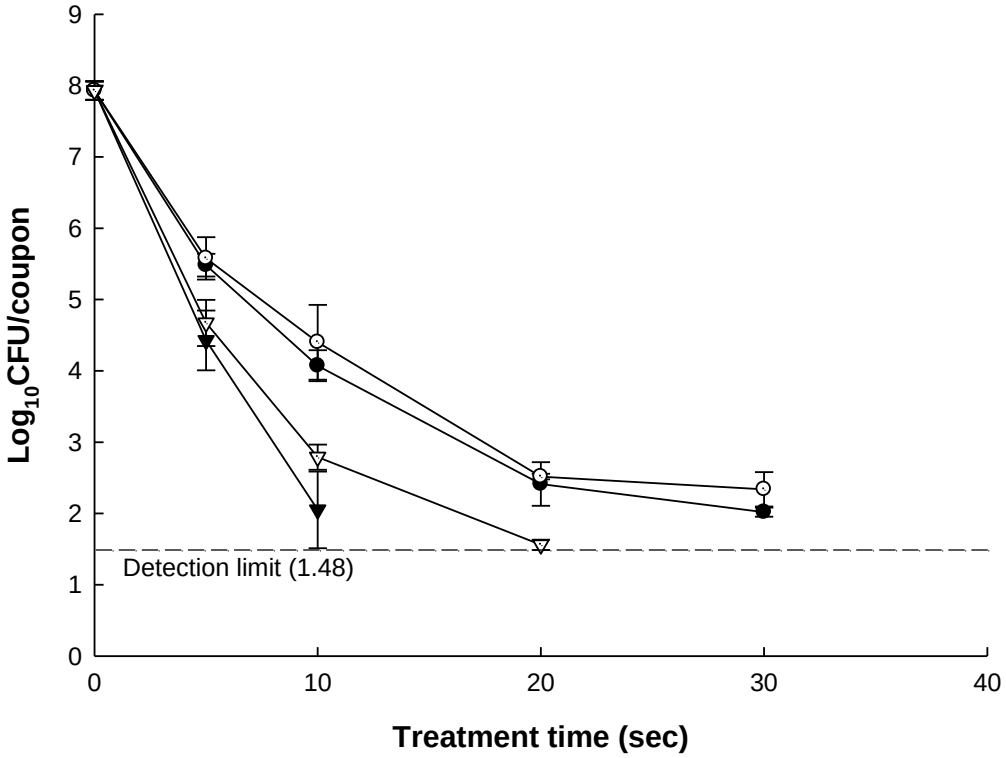

Fig. VI-4. Survival (log CFU/coupon) of biofilm cells of *Salmonella* Typhimurium formed on stainless steel coupons after steam pasteurization. ○, OV-XLD (75 ℃); ●, XLD (75 ℃); ▽, OV-XLD (85 ℃); ▼, XLD (85 ℃).

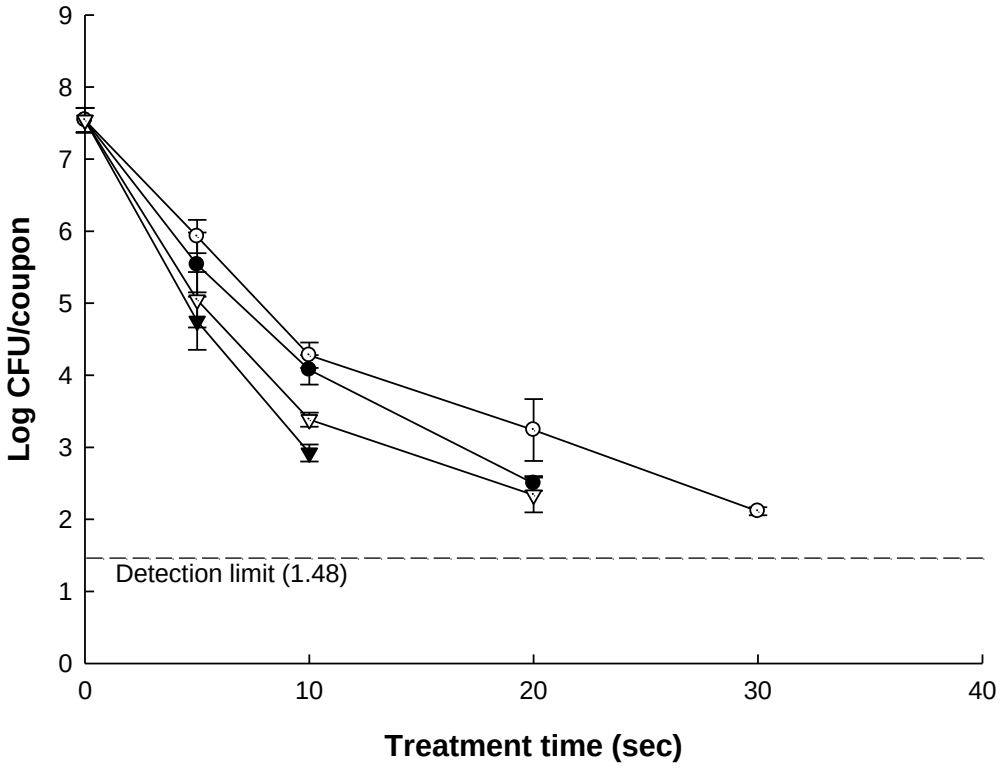

Fig. VI-5. Survival (log CFU/coupon) of biofilm cells of *Listeria monocytogenes* formed on stainless steel coupons after steam pasteurization. ○, OV-MOX (75 ℃); ●, MOX (75 ℃); ▽, OV-MOX (85 ℃); ▼, MOX (85 ℃).

Inactivation of biofilm cells formed on PVC. The initial biofilm cell counts of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on PVC were 7.68, 7.30, and 7.33 log CFU/coupon, respectively (Fig. VI-6, 7, and 8). Steam treatment led to less reduction of biofilm cells on PVC compared to those on stainless steel. Levels of biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were reduced by steam treatment at 75 ℃ for 50 s, showing 2.47, 2.57, and 2.04 log CFU/coupon reductions, respectively. Significant ($p < 0.05$) further reductions were observed with steam treatment at 85 ℃. Steam treatment at 85 ℃ for 50 s caused 3.61, 3.39, and 2.52 log CFU/coupon reductions, respectively. Similar to the results of stainless steel, significant ($p < 0.05$) differences between the levels of injured cells and healthy cells were observed as steam treatment time increased.

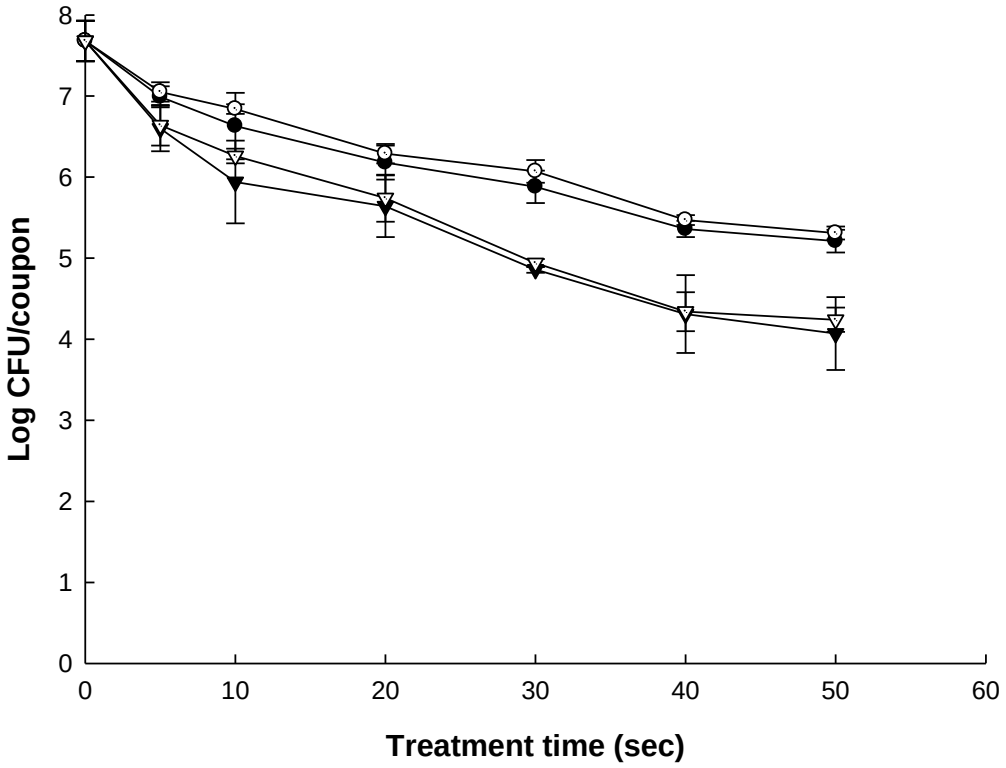

Fig. VI-6. Survival (log CFU/coupon) of biofilm cells of *Escherichia coli* O157:H7 formed on PVC coupons after steam pasteurization. ○, SPRAB (75 ℃); ●, SMAC (75 ℃); ▽, SPRAB (85 ℃); ▼, SPRAB (85 ℃).

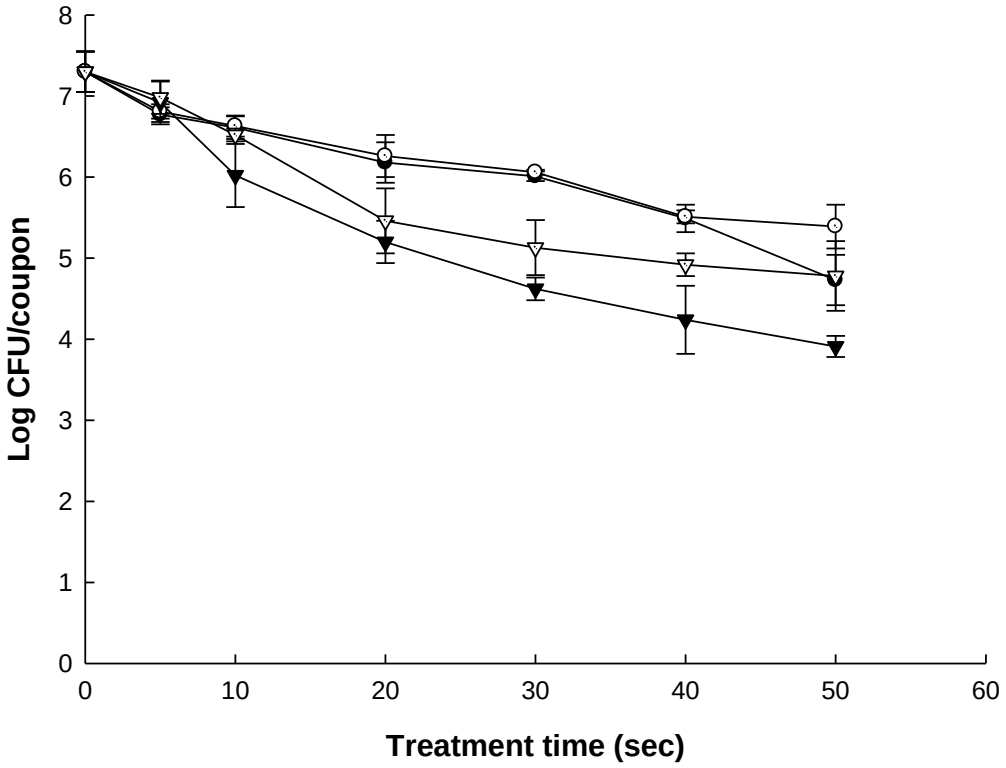

Fig. VI-7. Survival (log CFU/coupon) of biofilm cells of *Salmonella* Typhimurium formed on PVC coupons after steam pasteurization. ○, OV-XLD (75 ℃); ●, XLD (75 ℃); ▽, OV-XLD (85 ℃); ▼, XLD (85 ℃).

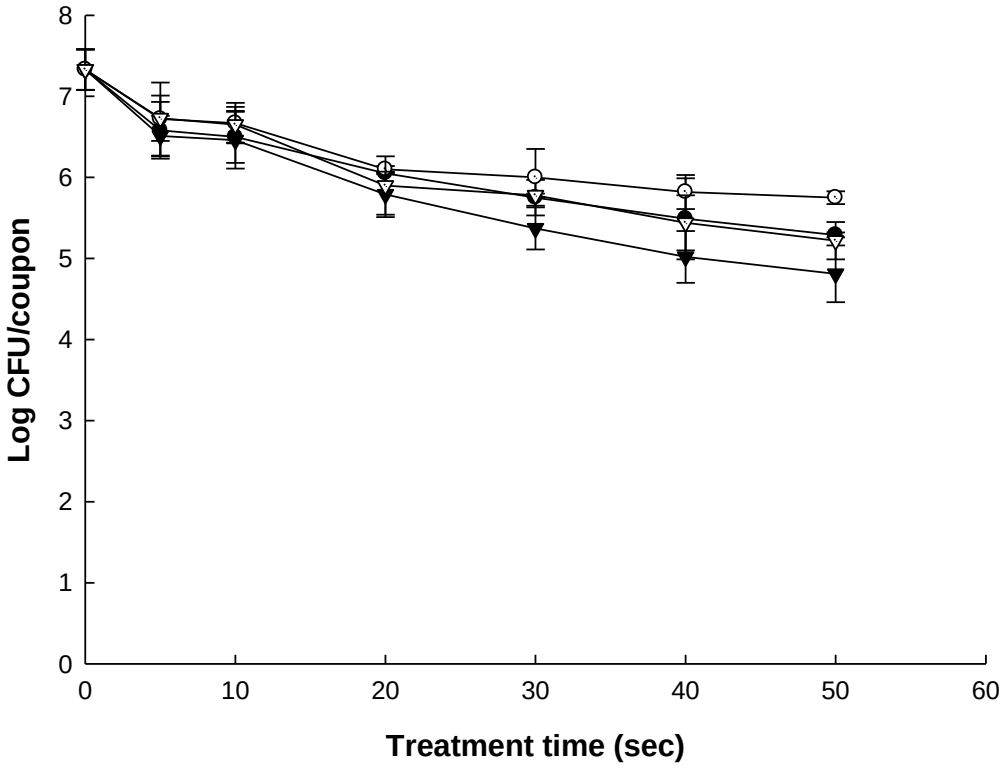

Fig. VI-8. Survival (log CFU/coupon) of biofilm cells of *Listeria monocytogenes* formed on PVC coupons after steam pasteurization. ○, OV-MOX (75 ℃); ●, MOX (75 ℃); ▽, OV-MOX (85 ℃); ▼, MOX (85 ℃).

VI-4.5. Discussion

Steam pasteurization for the decontamination of meat has been used extensively in the United States, Canada, Australia, and the European Union (EU) (Sheridan, 2004). The antimicrobial effect of steam on meat products has been evaluated and the data suggests that steam pasteurization may be effective in controlling meat contaminated with foodborne pathogens (McCann et al., 2006). Significant ($p < 0.05$) reductions in the counts of *Campylobacter* were observed on broiler carcasses exposed to steam at 90 ℃ for 12 s (Whyte et al., 2003). Murphy et al. (2006) reported that about 3 log CFU/cm^2 reduction of *L. monocytogenes* was achieved when frankfurters were treated with steam at 114 ℃ for 1.5 s. Steam treatment at 99 to 101 ℃ for 15 s reduced *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* populations on surfaces of freshly slaughtered beef by 3.53, 3.74, and 3.44 log CFU/cm^2, respectively (Phebus et al., 1997).

In the present study, steam pasteurization showed significant antimicrobial effect against biofilm cells of foodborne pathogens on stainless steel and PVC. Biofilm cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on stainless steel coupons were reduced to below the detection limit (1.48 log CFU/coupon) after steam treatment at 75 ℃ for 30 to 40 s, and at 85 ℃ for 20 to 30 s. Steam treatment caused less reduction in biofilm cells of three foodborne pathogens on PVC coupons. Levels of biofilm cells of three foodborne pathogens were reduced by 2.04 to 2.57

log CFU/coupon and 2.52 to 3.61 log CFU/coupon after 50 s of steam treatment at 75 ℃ and 85 ℃, respectively. Differences in microbial reduction patterns between stainless steel and PVC may be due to different thermal conductivity. When stainless steel and PVC coupons were treated with steam at 75 ℃, the temperature of stainless steel coupons reached 75 ℃ within 5 s, while PVC coupons reached this temperature within 15 s (data not shown). In this study, significant ($p < 0.05$) differences between the levels of injured cells and healthy cells were observed as steam treatment time increased. Because injured cells of foodborne pathogens are potentially as dangerous as their uninjured counterparts (Lee and Kang, 2001; McCleer and Rowe, 1995), sub-lethally injured foodborne pathogens could assume added significance following steam treatment. To completely inactivate biofilm cells of foodborne pathogens, a higher steam temperature than that evaluated in this study should be applied.

Other physical control methods have been evaluated to remove biofilms on food processing surfaces. Baumann et al. (2009) reported that ultrasound treatment (20 KHz, 120 W) for 60s at a distance of 2.54 cm from biofilm chips reduced levels of *L. monocytogenes* biofilms by 3.8 log CFU/ml. Nanostructured TiO_2 thin films on stainless steel and glass are an alternative means of disinfecting contaminated surfaces (Chorianopoulos et al., 2011). The biofilm cells of *L. monocytogenes* on glass were reduced by 3 log CFU/cm^2 when TiO_2 was activated by ultraviolet A light for 90 min. Gamma irradiation (3kGy) decreased the biofilm cells of *E. coli* formed

on polypropylene and polyester to below the detection limit (< 10^1 CFU/ml) (Byun et al., 2007). However, these methods cannot be applied to control biofilms in inaccessible areas in the food processing line, while steam pasteurization can be used for cleaning food processing surfaces.

Control of biofilms is an important issue for the food processing industry. This study showed that steam pasteurization was able to effectively inactivate biofilm cells of foodborne pathogens on stainless steel and PVC, which are common food processing surfaces. It may provide the food industry with a cleaning procedure for controlling biofilms of foodborne pathogens in food processing facilities. However, it is suggested that a post-removal procedure is needed to completely remove biofilm residues. Also, further study is required to increase the effectiveness of steam treatment to achieve desired safety limits on PVC surfaces.

VI-4.6. References

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Chapter VII.

Appendix : Development of a novel selective and differential medium for the isolation of *Listeria monocytogenes*

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VII-1. Abstract

A new medium (Lecithin and Levofloxacin - LL medium) is described for the isolation of *Listeria monocytogenes* from food samples. LL medium includes lecithin from soybeans for the detection of phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC) produced by *L. monocytogenes*. Levofloxacin is incorporated to inhibit the growth of microorganisms other than *L. monocytogenes*, especially *Bacillus cereus*, shown to possess PI-PLC and PC-PLC activity. *L. monocytogenes* produced white colonies with a halo on LL medium, whereas *L. innocua* appeared as white colonies without a halo. Levofloxacin at 0.20 mg/L completely inhibited the growth of *B. cereus*, while the growth of *L. monocytogenes* was unaffected. In the second phase of the study, the sensitivity and the specificity of LL medium was compared to modified oxford (MOX) and two chromogenic media (Brilliance *Listeria* agar and CHROMagar *Listeria*) using a total of 250 food samples. From 200 unspiked food samples, the specificity of LL medium (96.0%) was superior to that of MOX (72.0%), and similar to specificities of Brilliance *Listeria* agar (96.5%) and CHROMagar *Listeria* (94.5%). From 50 spiked food samples, LL medium and Chromagar *Listeria* represented the highest sensitivities (96.0%), followed by Brilliance *Listeria* agar (92.0%) and MOX (54.0%). Also, LL medium showed the highest confirmation rate (98.8%), followed by Brilliance *Listeria* agar (98.7%),

Chromagar *Listeria* (98.3%), and MOX (52.0%). On the basis of its good specificity and cost effectiveness, LL medium is useful for the isolation of *L. monocytogenes* from food samples.

VII-2. Introduction

The gram-positive foodborne pathogen *Listeria monocytogenes* is a significant public health and food safety concern worldwide (Pradhan et al., 2011; Todd and Notermans, 2011). Infection of pregnant women, infants, the elderly, and immunosuppressed individuals with this pathogen can lead to listeriosis, a disease condition that can induce severe illnesses and relatively high mortality rates (Painter and Slutsker, 2007; Swaminathan and Gerner-Schmidt, 2007). Infection has been associated with foods such as cheese, meat, milk, vegetables, and fish (Dalton et al., 1997; De Valk et al., 2001; Lunden et al., 2004; Makino et al., 2005; Tham et al., 2000). Thus, effective methods for the isolation of *L. monocytogenes* from various foods are important to ensure food quality and safety.

A wide variety of selective and differential media have been developed for this purpose, including PALCAM, Oxford, and modified oxford (MOX) (Curtis et al., 1989; McClain and Lee, 1989; van Netten et al., 1989). *L. monocytogenes* growing on these media is detected by the action of esculinase cleaving esculin (McLauchlin, 1987). However, this metabolic enzyme is common to all *Listeria* species, so it does not distinguish *L. monocytogenes* from other non-pathogenic species of *Listeria* (Hegde et al., 2007; Leclercq, 2004). Therefore, subculture of multiple colonies is required to confirm the species which takes at least a further 2 days (Greenwood et al., 2005). *Listeria innocua* in particular is often detected in food stuffs and food

environments (Johansson, 1998). Also, *L. innocua* has been reported to grow faster than *L. monocytogenes* in enrichment broth, which makes detection of *L. monocytogenes* more difficult (Zitz et al., 2011).

Several chromogenic media including BBL™ CHROMagar™ *Listeria*, ALOA, Rapid'L. mono Agar, CHROMagar™ *Listeria*, and Oxoid chromogenic *Listeria* agar (OCLA) have been introduced for the differentiation of *L. monocytogenes* from other *Listeria* spp. (Carles et al., 1997; Hegde et al., 2007; Ottaviani et al., 1997; Restaino et al., 1999; Willis et al., 2006). The detection of *L. monocytogenes* by chromogenic media usually involves cleavage of the substrate, 5-bromo-4-chloro-3-indoxl- β-D-glucopyranoside by β-D-glucosidase produced by *Listeria* spp., combined with L-α-phosphatidyl-inositol for the detection of phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC) (Hegde et al., 2007 15, ; Leclercq, 2004; Reissbrodt, 2004; Stessl et al., 2009). PI-PLC and PC-PLC, the major virulence factors, are only produced by pathogenic *L. monocytogenes* and *L. ivanovii* (Leclercq, 2004, Mengaud et al., 1991). Chromogenic substrates have proven to be an efficient tool, utilizing specific enzymatic reactions of certain bacteria (Pradhan et al., 2011). However, chromogenic media can be expensive, and may not be suitable for routine laboratory use (Willis et al., 2006). Also some *Bacillus* spp., especially *Bacillus cereus* and *Staphylococcus aureus* are β-D glucosidase, PI-PLC, and PC-PLC positive, thus they could grow on chromogenic media and produce colonies similar to those of *L.*

monocytogenes (Daugherty and Low, 1993; Ghelardi et al., 2002; Kloos and Wolfshohl, 1982; van Netten and Kramer, 1992; Vlaemynck et al., 2000; Willis et al., 2006).

Recognizing the limits of currently used selective and differential media, it is desirable to develop an alternative medium with improved specificity while maintaining cost effectiveness. The aim of this study was to develop a new selective and differential medium for the detection of *L. monocytogenes* (LL medium), and to compare the specificity and the sensitivity of LL medium with conventional MOX and two chromogenic media using stock cultures and food samples.

VII-3. Materials and Methods

Stock cultures. The test bacteria used in this study (Table VII-1 and 2) were obtained from the American Type Culture Collection (ATCC), the National Culture Collection for Pathogens (NCCP; Osong, Korea), Korean Collection for Type Culture (KCTC), or the bacterial culture collection of the Food Hygiene Laboratory at Seoul National University (SNCC; Seoul, Korea). They were stored frozen at -80 ℃.

Growth of test bacteria on basal medium containing different concentrations of levofloxacin. To determine optimum concentration of antibiotics, the growth of bacteria on basal medium containing four different concentrations of levofloxacin was tested. The ingredients of basal medium for this study were as follows: 8.9 g of pancreatic digest of casein (Neogen, Lansing, MI, USA), 4.4 g of protease peptone (Difco, Sparks, MD, USA), 4.4 g of yeast extract (Difco), 2.7 g of beef heart infusion (Difco), 4.4 g of sodium chloride (Samchun Chemical Co. Ltd., Pyeongtaeksi, Korea), 15.0 g of lithium chloride (Samchun Chemical), and 15.0 g of Bacto^{TM} agar (Difco) per liter. The ingredients were added to distilled water, and sterilized at 121 ℃ for 15 min. After sterilization, the medium was tempered to 48 ℃, and 10.0 mg of colistin sulfate (Difco) and 20.0 mg of moxalactam (Difco) were added to the basal medium. Prepared basal medium was supplemented with levofloxacin (Sigma-Aldrich, St Louis, MO, USA) at concentrations from 0.10 mg/L

to 0.25 mg/L at 0.05 mg/L increments. Modified oxford (MOX) was prepared with Oxford agar base (Difco) supplemented with modified oxford antimicrobic supplement (Difco). The test bacteria (Table VII-1) were incubated in 10 ml of tryptic soy broth (TSB; Difco) at 37 ℃ for 18 h. After incubation, one loopful of each strain was streaked onto MOX and each basal medium containing different concentrations of levofloxacin by the semi-quantitative three-loop technique (Stessl et al., 2009). This involves streaking three times on each test medium using a sterile loop to make dilutions of that original streak. After incubation for 48 h at 37 ℃, the amount of growth on the agar is then reported semi-quantitatively as many (+++), moderate (++), or few (+), respectively, depending on how far out from the inoculum site colonies appear. The test bacteria that grow in all streaked areas would be reported as many (+++). The test bacteria that only grow in the original streak area would be reported as few (+).

Preparation of LL medium. The ingredients of basal medium were added to 950 ml of distilled water and sterilized at 121 ℃ for 15 min. After sterilization, the medium was tempered to 48 ℃ and 10.0 mg of colistin sulfate and 20.0 mg of moxalactam were added to the basal medium. Levofloxacin (0.2 mg) was also supplemented to the basal medium. Five gram of soya lecithin (Solae, St Louis, MO, USA) was dissolved in 50 ml of distilled water and sterilized at 121 ℃ for 15 min, and added to the prepared medium. The medium was poured into 9-cm-diameter petri dishes.

Recovery of *L. monocytogenes* on MOX and LL medium. Fifteen *L. monocytogenes* strains were grown in 10 ml of TSB at 37 ℃ for 18 h. The cultures were tenfold serially diluted with 9 ml of buffered peptone water (BPW; Difco), and 0.1 ml of diluents (10^{-4}-10^{-7}) were plated onto MOX and LL medium. Colony counts were recorded after incubation at 37 ℃ for 48 h. This experiment was independently repeated three times.

Evaluation of the performance of LL medium with stock cultures. The performance of LL medium was evaluated using stock cultures in comparison with MOX, Brilliance *Listeria* agar (Oxoid, Ltd., Basingstoke, UK), and CHROMagar *Listeria* (CHROMagar, Paris, France). Twenty-nine *L. monocytogenes* strains (Table VII-1) were selected for the inclusivity test while 76 non- *L. monocytogenes* strains were used for exclusivity test. The latter consisted of *Listeria ivanovii* (*n* = 1), *L. innocua* (*n* = 4), *Listeria welschimeri* (*n* = 1), *Listeria seeligeri* (*n* = 1), *Listeria grayi* (*n* = 1), *B. cereus* (*n* = 24), *Bacillus circulans* (*n* = 3), *Staphylococcus aureus* (*n* = 10), *Staphylococcus epidermidis* (*n* = 2), *Staphylococcus xylosus* (*n* = 1), unidentified isolates from MOX plates (*n* = 10), *Escherichia coli* (*n* = 8), *Klebsiella pneumonia* (*n* = 4), *Yersinia enterocolitica* (*n* = 2), *Hafnia alvei* (*n* = 3), and *Citrobacter freundii* (*n* = 1). The test bacteria were grown in 10 ml of TSB at 37 ℃ for 18 h. After incubation, one loopful of each strain was streaked onto each medium, and incubated at 37 ℃ for 24-48 h. The growth and colony morphology were observed after incubation.

Evaluation of the specificity and the sensitivity of LL medium using spiked and unspiked food samples. The conventional culture method described by the U.S. Food and Drug Administration (FDA) (Hitchins and Jinneman, 2011) was used for the microbiological analysis of spiked and unspiked food samples. In this study, a total 250 food samples (200 unspiked food samples, 25 food samples spiked with *L. monocytogenes*, and 25 food samples spiked with *L. monocytogenes* and *L. innocua*) were tested for evaluation of the specificity and the sensitivity of LL medium. These food samples consisting of ground beef, ground pork, deli meat, smoked ham, sausage, luncheon meat, mixed salad, mixed sprouts, potato, lettuce, carrots, spinach, sweet potatoes, celery, radish, milk, cheddar cheese, and shrimp were purchased from local retail markets (Seoul, Korea). All foods which were used in the spiked food sample test were previously screened to ensure they were *Listeria*-free. To prepare spiked food samples, each strain of *L. monocytogenes* (ATCC 19114, 19115, and 15313) and *L. innocua* (SNCC 1, 2, and 3) was grown in 10 ml of TSB at 37 ℃ for 18 h. Twenty-five subsamples (25 g or 25 ml) of each food were inoculated with 3 different *L. monocytogenes* strains at an inoculum level of 50–100 CFU/25 g or ml. Also, 25 subsamples of each food were inoculated with 3 different *L. monocytogenes* strains and 3 different *L. innocua* strains at an inoculum level of 50–100 CFU/25 g or ml. Spiked and unspiked food samples were introduced into a sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of buffered Listeria enrichment broth (BLEB; Difco) and homogenized with a

stomacher (EASY MIX, AES Chemunex, Rennes, France) for 1 min. After pre-enrichment at 30 ℃ for 4 h, acriflavine (final concentration: 10 mg/liter) (Sigma-Aldrich), nalidixic acid (final concentration: 40 mg/liter) (Sigma-Aldrich), and cycloheximide (final concentration: 50 mg/liter) (Sigma-Aldrich) were added, and incubated at 30 ℃ for 44 h. After enrichment, one loopful of each sample was streaked onto LL medium, MOX, Brilliance *Listeria* agar, and CHROMagar *Listeria*, and incubated at 37 ℃ for 24-48 h. After incubation, up to 5 colonies suspected of being *L. monocytogenes* on these media were selected for identification. Bacterial colonies were identified using the API *Listeria* test (bioMérieux, Marcy l'Etoile, France) and the Vitek 2 system (bioMérieux). Colonies suspected of being *L. monocytogenes* were defined as white colonies with a halo on LL medium, black colonies with a black halo on MOX, blue/green colonies with halo on Brilliance *Listeria* agar, and blue colonies with white halo on CHROMagar *Listeria*.

The specificity was evaluated by calculating the proportion of *L. monocytogenes*-negative samples correctly found to be negative (i.e., those that did not appear as presumptive *L. monocytogenes* colonies). The sensitivity was calculated as the proportion of *L. monocytogenes*-positive samples correctly found to be positive (Willis et al., 2006). The confirmation rate was determined as the ratio of the number of confirmed *L. monocytogenes* colonies (C), to the number of total suspect colonies tested (S), expressed as : [C/S] × 100% (Hegde et al., 2007).

Statistical analyses. The significance of differences in colony counts was calculated by the Student's t test with a significance level of $p < 0.05$, using Microsoft® Excel. The significance of differences in the specificity and the sensitivity was calculated by the McNemar tests with a significance level of $p < 0.05$, using the SPSS software package (SPSS, Chicago, USA).

VII-4. Results

Growth of test bacteria on basal medium containing different concentrations of levofloxacin. The growth of *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *Listeria welschimeri*, *Listeria seeligeri*, *Listeria grayi*, and *B. cereus* was observed on basal medium containing different concentrations of levofloxacin (Table VII-1). Three strains of *L. monocytogenes* (NCCP 10810, SNCC 1, and SNCC 15) and *L. ivanovii* only grew in the original streaking area of basal medium containing 0.25 mg/L of levofloxacin. Basal medium containing 0.20 mg/L of levofloxacin achieved satisfactory growth (++, +++) for all *L. monocytogenes* strains, *L. ivanovii*, *L. innocua*, and *L. welschimeri* tested. *L. seeligeri* did not grow on all tested basal medium, and basal medium containing 0.10 mg/L of levofloxacin was the only medium that supported growth of *L. grayi*. Basal medium containing 0.20 mg/L of levofloxacin completely inhibited the growth of all *B. cereus* strains. Levofloxacin (0.15 mg/L and 0.10 mg/L) did not completely inhibit the growth of *B. cereus*.

Table VII-1. Growth of *Listeria* spp. and *B. cereus* on medium with different levofloxacin concentrations.

Species	Strain	The amount of growth on medium with levofloxacin concentration (mg/L) of			
		0.25	0.20	0.15	0.10
L. monocytogenes	ATCC 19114	+++	+++	+++	+++
L. monocytogenes	ATCC 19115	+++	+++	+++	+++
L. monocytogenes	ATCC 15313	+++	+++	+++	+++
L. monocytogenes	NCCP 10810	+	+++	+++	+++
L. monocytogenes	NCCP 10811	+++	+++	+++	+++
L. monocytogenes	NCCP 10943	+++	+++	+++	+++
L. monocytogenes	SNCC 1	+	++	+++	+++
L. monocytogenes	SNCC 2	+++	+++	+++	+++
L. monocytogenes	SNCC 3	+++	+++	+++	+++
L. monocytogenes	SNCC 4	+++	+++	+++	+++
L. monocytogenes	SNCC 5	+++	+++	+++	+++
L. monocytogenes	SNCC 6	+++	+++	+++	+++
L. monocytogenes	SNCC 7	+++	+++	+++	+++
L. monocytogenes	SNCC 8	+++	+++	+++	+++
L. monocytogenes	SNCC 9	+++	+++	+++	+++
L. monocytogenes	SNCC 10	+++	+++	+++	+++
L. monocytogenes	SNCC 11	+++	+++	+++	+++
L. monocytogenes	SNCC 12	+++	+++	+++	+++
L. monocytogenes	SNCC 13	+++	+++	+++	+++
L. monocytogenes	SNCC 14	+++	+++	+++	+++
L. monocytogenes	SNCC 15	+	++	+++	+++
L. monocytogenes	SNCC 16	+++	+++	+++	+++
L. monocytogenes	SNCC 18	+++	+++	+++	+++
L. monocytogenes	SNCC 19	+++	+++	+++	+++
L. monocytogenes	SNCC 21	+++	+++	+++	+++
L. monocytogenes	SNCC 22	++	+++	+++	+++
L. monocytogenes	SNCC 23	+++	+++	+++	+++
L. monocytogenes	SNCC 24	++	+++	+++	+++
L. monocytogenes	SNCC 25	+++	+++	+++	+++
L. ivanovii	NCCP 10953	+	+++	+++	+++
L. innocua	SNCC 1	+++	+++	+++	+++
L. innocua	SNCC 2	+++	+++	+++	+++
L. welschimeri	NCCP 10965	++	+++	+++	+++
L. seeligeri	NCCP 10966	-[a]	-	-	-

L. grayi	NCCP 10879	-	+	+	++
B. cereus	ATCC 10876	-	-	-	+
B. cereus	ATCC 13061	-	-	+	++
B. cereus	ATCC 14579	-	-	-	+
B. cereus	NCCP 11308	-	-	+	+
B. cereus	NCCP 10109	-	-	+	+
B. cereus	NCCP 11307	-	-	+	+
B. cereus	NCCP 11306	-	-	+	+
B. cereus	NCCP 11313	-	-	-	-
B. cereus	NCCP 11309	-	-	-	-
B. cereus	NCCP 10084	-	-	-	-
B. cereus	NCCP 11310	-	-	-	-
B. cereus	NCCP 11311	-	-	-	-
B. cereus	SNCC 1	-	-	-	+
B. cereus	SNCC 2	-	-	-	+
B. cereus	SNCC 3	-	-	-	-
B. cereus	SNCC 4	-	-	-	-
B. cereus	SNCC 5	-	-	-	-
B. cereus	SNCC 6	-	-	-	-
B. cereus	SNCC 7	-	-	-	-
B. cereus	SNCC 8	-	-	-	-
B. cereus	SNCC 9	-	-	-	-
B. cereus	SNCC 10	-	-	-	-
B. cereus	SNCC 11	-	-	-	-
B. cereus	SNCC 12	-	-	-	-

[a] -, no growth; +, grow in the original streak area; ++, grow in 2nd streaking area; +++, grow in all streaked areas.

Recovery of *L. monocytogenes* on MOX and LL medium. Bacterial counts on LL medium were not significantly ($p > 0.05$) different from those on MOX (data not shown). On MOX and LL medium, colonies did not always have the typical appearance after 24 h incubation (data not shown). At 48 h, all *L. monocytogenes* strains developed the correct morphology on these media (black with a black halo and white with a halo, respectively).

Evaluation of the performance of LL medium with stock cultures. All tested *L. monocytogenes* strains produced typical *L. monocytogenes* colonies on each tested medium (inclusivity 100%) (Table VII-2). The best exclusivity accomplished by Brilliance *Listeria* agar and LL medium. Only *L. ivanovii* ($n = 1$) produced presumptive *L. monocytogenes* colonies on these media, and these two media achieved an exclusivity of 98.7%. CHROMagar *Listeria* achieved an exclusivity of 96.1%. One strain of *L. ivanovii* and two strains of *B. cereus* produced colonies similar to *L. monocytogenes* on CHROMagar *Listeria*. MOX showed an exclusivity of 77.6%. *L. innocua* ($n = 4$), *L. welschimeri* ($n = 1$), *L. grayi* ($n = 1$), and unidentified isolates ($n = 10$) produced black colonies with a black halo on MOX indistinguishable from those of *L. monocytogenes*. All tested media did not support the growth of *L. seeligeri*. Fig. VII-1 shows colonies of *L. monocytogenes*, *L. ivanovii*, and *L. innocua* formed on MOX and LL medium. *L. monocytogenes* (Fig. VII-1A) and *L. ivanovii* (Fig. VII-1B) produced typical black colonies with a black halo on MOX. However, *L. innocua* (Fig. VII-1C) produced colonies similar to

those of *L. monocytogenes* and *L. ivanovii*. *L. monocytogenes* (Fig. VII-1D) and *L. ivanovii* (Fig. VII-1E) appeared as white colonies with a halo on LL medium, whereas *L. innocua* produced white colonies without a halo (Fig. VII-1F).

Table VII-2. Evaluation of the performance of each medium with stock cultures.

	MOX	Brilliance *Listeria* agar	Chromagar *Listeria*	LL medium
L. monocytogenes (*n* = 29)	29	29	29	29
True positive	29	29	29	29
False negative	0	0	0	0
Non-*L. monocytogenes* (*n* = 76)				
False positive	17	1	3	1
True negative	59	75	73	75
Accuracy (%)[a]	85.2	99.0	98.5	99.0
Exclusivity (%)[b]	77.6	98.7	96.1	98.7
Inclusivity (%)[c]	100	100	100	100

[a] (True positive+true negative)/total×100.
[b] True negative/(true negative+false positive)×100.
[c] True positive/(true positive+false negative) ×100.

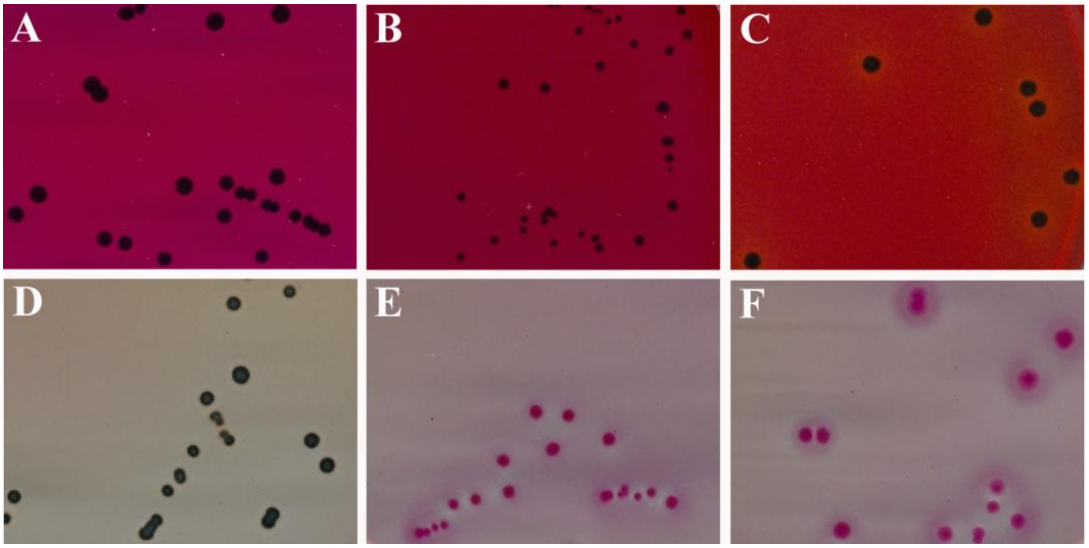

Fig. VII-1. Colonies produced by *S*. Typhimurium, *C. freundii*, and *P. mirabilis* on XLD (A, B, and C) and XA medium (D, E and F). Colonies of *S*. Typhimurium (A), *C. freundii* (B), and *P. mirabilis* (C) appeared as black colonies on XLD. On XA medium, *S*. Typhimurium (D) produced black colonies, whereas *C. freundii* (E) and *P. mirabilis* (F) produced pink colonies.

Evaluation of the specificity and the sensitivity of LL medium using spiked and unspiked food samples. Table VII-3 represents results of the bacteriological analysis of unspiked food samples. The number of negative samples was equal to the number of total food samples, because no *L. monocytogenes* strains were isolated on all tested media from 200 unspiked food samples. There were no significant differences among specificities of Brilliance *Listeria* agar, CHROMagar *Listeria*, and LL medium. Specificities of Brilliance *Listeria* agar, CHROMagar *Listeria*, and LL medium were 96.5, 94.5, and 96.0%, respectively. Specificities of these three media were superior to the specificity of MOX (72.0%). A total of 56, 7, 11, and 8 false-positive results were found on MOX, Brilliance *Listeria* agar, CHROMagar *Listeria*, and LL medium, respectively. From 50 spiked food samples, Chromagar *Listeria* and LL medium represented the highest sensitivities (96.0%), followed by Brilliance *Listeria* agar (92.0%) and MOX (54.0%) (Table VII-4). Also, LL medium showed highest confirmation rate (98.8%), followed by Brilliance *Listeria* agar (98.7%), Chromagar *Listeria* (98.3%), and MOX (52.0%).

Table VII-3. The specificity of LL medium compared with that of MOX, Brilliance *Listeria* agar, and CHROMagar *Listeria* on the microbiological analysis of 200 unspiked food samples.

Medium	No. of true-negative results	No. of false-positive results	Specificity (%)[a]
MOX	144	56	72.0
Brilliance *Listeria* agar	193	7	96.5
Chromagar *Listeria*	189	11	94.5
LL medium	192	8	96.0

[a] (No. of true-negative results on the medium/No. of negative samples)×100.

Table VII-4. Evaluation of efficacy of each medium for the isolation of *L. monocytogenes* from 50 spiked food samples.

Media	Sensitivity[a] (%)	Confirmation rate[b] (%)
MOX	54.0	52.0 (130/250)
Brilliance *Listeria* agar	92.0	98.7 (227/230)
Chromagar *Listeria*	96.0	98.3 (236/240)
LL medium	96.0	98.8 (237/240)

[a] No. of true positives/(No. of true positives+No. of false negatives) × 100.
[b] Percentage of suspect colonies tested and confirmed positive for *L. monocytogenes*.

VII-5. Discussion

Plating on selective and differential media, such as PALCAM, Oxford, MOX, and chromogenic agar, after pre-enrichment followed by selective enrichment in BLEB has been recommended for the isolation of *L. monocytogenes* from foods by the U.S. FDA (Hitchins and Jinneman, 2011). However, PALCAM, Oxford, and MOX, which use esculinase activity of *Listeria* spp., cannot differentiate *L. monocytogenes* from other *Listeria* spp. Also, several chromogenic media which use chromogenic compounds such as 5-bromo-4-chloro-3-indoxl- β-D-glucopyranoside and L-α-phosphatidyl-inositol are expensive (Willis et al., 2006).

Oxoid chromogenic Listeria agar (OCLA) contains soya lecithin instead of phosphatidyl inositol for the differentiation of *L. monocytogenes* from other *Listeria* spp. (Willis et al., 2006). The results suggest that soya lecithin is an appropriate alternative to phosphatidyl inositol. Soya lecithin is a cheap and easily available product, and could be used for the detection of PI-PLC and PC-PLC activity (da Silva Malheiros et al., 2010; Willis et al., 2006). In this study, LL medium contains soya lecithin for the differentiation of *L. monocytogenes* from other *Listeria* spp. *L. monocytogenes* and *L. ivanovii* produced distinct colonies (white colonies with a halo) from those of other *Listeria* spp. on LL medium. Although *L. ivanovii* is less frequently encountered in foods, it is important to detect this organism because some cases of human infection by *L. ivanovii* have been reported (Guillet et al., 2010).

Some *Bacillus* spp., especially *Bacillus cereus*, are β-D glucosidase, PI-PLC, and PC-PLC positive (Ghelardi et al., 2002, van Netten et al., 1992; Vlaemynck et al., 2000), thus they could grow on chromogenic media and produce colonies similar to those of *L. monocytogenes* (Stessl et al., 2009; Vlaemynck et al., 2000). Therefore, it is necessary to inhibit growth of *B. cereus* to ensure specificity of the selective medium. In a previous study (Luna et al., 2007) on 42 isolates of *B. cereus*, the MIC_{50} and MIC_{90} of levofloxacin were 0.125 and 0.25 mg/L, respectively. Forty-two strains of *L. monocytogenes* were more resistant to levofloxacin, the MIC_{50} and MIC_{90} of levofloxacin were 0.5 and 1 mg/L, respectively (Salas et al., 2008). LL medium contains levofloxacin in addition to colistin sulfate and moxalactam to inhibit the growth of *B. cereus*. Levofloxacin concentration from 0.25 to 0.20 mg/L completely inhibited growth of all *B. cereus* strains tested. LL medium includes 0.20 mg/L of levofloxacin, because the growth of some *L. monocytogenes* strains and L. *ivanovii* was inhibited on basal medium containing 0.25 mg/L of levofloxacin. The recovery of *L. monocytogenes* on LL medium was compared with MOX to evaluate the effect of additional antibiotics on the sensitivity of the medium (data not shown). Counts of *L. monocytogenes* on LL medium reached approximately the same level as those on MOX, thus levofloxacin could be used as a selective agent in LL medium.

In this study, the specificity and the sensitivity of LL medium were compared with conventional MOX and two chromogenic media (Brilliance *Listeria* agar and

Chromagar *Listeria*) using stock cultures and food samples. Brilliance *Listeria* agar (formerly OCLA) contains soya lecithin instead of phosphatidyl inositol. LL medium completely suppressed the growth of non-*Listeria* gram-positive and gram-negative bacteria (Table VII-2). LL medium also inhibited the growth of *S.* aureus, *S. epidermidis*, and *S. xylosus*. *Staphylococcus* spp. which have PI-PLC and PC-PLC activities can give the appearance of false positive colonies on chromogenic medium which contains soya lecithin instead of phosphatidyl inositol (Daugherty and Low, 1993; Low, 1981; Willis et al., 2006). The growth of some unidentified bacteria isolated from food samples and producing black colonies with a black halo on MOX were also completely inhibited on LL medium. Brilliance *Listeria* agar and Chromagar *Listeria* also successfully inhibited the growth of non-*Listeria* gram-positive and gram-negative bacteria, but two *B. cereus* strains formed colonies similar to those of *L. monocytogenes* on CHROMagar *Listeria*.

When 200 unspiked food samples were examined, no *L. monocytogenes* was isolated on all media tested. The incidence of *L. monocytogenes* from domestic and imported foods in Korea was lower than that reported in previous studies from other countries (Baek et al., 2000). Of 1,537 domestic and imported food products, *L. monocytogenes* was isolated from 122 food samples (7.9%). Also Jo et al. (2011) reported *L. monocytogenes* was not isolated from 96 samples of fresh-cut produce or organic vegetables. *L. innocua* comprised the majority of presumptive *Listeria* colonies on MOX (data not shown). Especially, *L. innocua* was frequently detected

in beef and pork samples. When 50 spiked food samples were tested, the sensitivity and the confirmation rate of MOX were much lower than those of LL medium, Brilliance *Listeria* agar, and Chromagar *Listeria*. From 25 samples which were spiked with *L. monocytogenes* and *L. innocua*, colonies of *L. monocytogenes* were hardly differentiated from those of *L. innocua* on MOX. *L. innocua* has been known to frequently outgrow *L. monocytogenes* in enrichment broth (Zitz et al., 2011). The ability to detect small numbers of *L. monocytogenes* colonies amongst colonies of *L. innocua* is an important advantage of LL medium.

In conclusion, LL medium presented in this study has higher specificity and sensitivity than MOX. Especially, LL medium easily differentiates *L. monocytogenes* and *L. ivanovii* from *L. innocua* that frequently produce false-positive results on conventional PALCAM, Oxford, and MOX. Also, LL medium showed similar specificity and sensitivity to Brilliance *Listeria* agar and Chromagar *Listeria* with reduced cost. The cost of LL medium ($33.70/liter) is lower than that of chromogenic media (Brilliance *Listeria* agar, $63.98/liter; Chromagar *Listeria*, $63.50/liter). LL medium could also inhibit the growth of PI-PLC and PC-PLC positive bacteria such as *B. cereus* and *Staphylococcus* spp. which cause false positive result on some chromogenic media. Due to the good specificity of LL medium, labor and time necessary for further confirmation tests may be applied to fewer colonies. Also, cost-effectiveness of LL medium would make it suitable for routine laboratory use. Although further study is required to evaluate the growth of

more *Listeria* spp. on LL medium, LL medium may provide a valuable addition to the array of selective media available for the detection of *L. monocytogenes* from foods.

VII-5. References

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국문 초록

이산화염소(ClO_2)는 근래 주목 받는 비가열 살균 기술 중 하나로 강한 산화력을 바탕으로 넓은 살균 범위를 나타낸다. 이산화염소의 효과는 pH 와 유기물질의 영향을 크게 받지 않으며, 이산화염소의 살균 기작은 미생물의 단백질 합성 저해 및 세포막 손상을 통한 세포의 투과성 증가이다. 이산화염소 가스는 수용액 상태의 이산화염소에 비해 높은 침투성을 가져 더 뛰어난 살균 효과를 보인다고 알려져 있으며 식품의 저장 및 운송 과정 중 적용 가능하다는 장점을 지닌다. 여러 가지 연구를 통해 이산화염소 가스의 식품 및 기구 표면 내 식중독 균 제어 효과가 보고된 바 있지만, 이산화염소 가스의 살균 효과에 영향을 미치는 요인들에 대한 연구는 부족한 실정이다. 또한, 이산화염소 가스와 다른 살균 기술과의 병행처리 연구도 많이 이루어지지 않고 있다.

본 연구의 세부적인 목표는, 1) 상대습도, 샘플의 표면 특성, 온도 조건이 이산화염소 가스의 식중독 균 (*Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*) 제어 효과에 미치는 영향 규명, 2) 이산화염소 가스와 자외선, 에어로졸 살균소독제 및 건열 처리의 병행을 통한 식중독 균 제어 효과 규명, 3) 서방성 이산화염소 가스 방출 조성물 개발이다.

시금치와 토마토 표면에 *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* 를 접종한 후, 이산화염소 가스 (1, 5, 10, 30, 50 ppmv)를 각각 다른 상대습도 조건 (50, 70, 90%)에서 최대 20 분간 처리하였다. 이산화염소 가스의 농도와 처리 시간이 증가할수록, 상대습도 조건에 따라 저감화 정도에 유의적인 차이가 관찰되었다 ($p < 0.05$). 전반적으로 시금치에서는 50%와 70%의 상대습도 조건에서의 저감화가 유의적인 차이를 나타내지 않았다 ($p > 0.05$). 시금치에 50 ppmv 의 이산화염소 가스를 20 분간 처리하였을 때, 50% 상대습도 조건에서는 1.25~1.78 log 저감화가 나타났으며 70% 상대습도 조건에서는 2.02~2.54 log 저감화가 나타났다. 이에 비해, 90% 상대습도 조건에서는 50 ppmv 이산화염소 가스를 15 분간 처리하였을 때 세 가지 병원균이 모두 검출한계 (1 log CFU/g) 이하로 저감화 되었다. 토마토의 경우 30 ppmv 이산화염소 가스를 20 분간 처리하였을 때 50% 상대습도 조건에서는 1.22~1.52 log 저감화가 나타났고, 70% 상대습도 조건에서는 15 분 처리 후 세 가지 병원균 모두 검출한계 (0.48 log CFU/cm^2) 이하로 저감화 되었다. 또한 90% 상대습도 조건에서는 10 분 처리 후 세 가지 병원균 모두 검출한계 (0.48 log CFU/cm^2) 이하로 저감화 되었다. 이산화염소 가스 처리에 따른 품질 변화를 확인하였을 때, 30 ppmv 이산화염소 가스 처리 시까지는 7 일간의 저장기간 동안 색과

텍스쳐 (texture)의 변화가 관찰되지 않았다.

샘플의 표면 특성이 이산화염소 가스의 식중독 균 제어 효과에 미치는 영향을 평가하기 위해 선별된 농산물 및 기구 표면의 소수성 (hydrophobicity) 및 거칠기 (roughness)를 접촉각 및 백색광간섭계 (white light scanning interferometry) 측정을 통해 각각 파악하였다. 선별된 농산물 (당근, 케일, 양배추, 시금치, 사과, 토마토, 파프리카) 및 기구 표면 (테플론, 실리콘, 고무, PVC, 스테인레스 스틸 2B, 스테인레스 스틸 No.4, 유리)에 세 가지 병원균을 접종한 후 20 ppmv 이산화염소 가스를 15 분간 처리하였다. 농산물 및 기구 표면의 접촉각은 세 가지 병원균의 저감화 정도와 높은 부적관계 (negative correlation)를 나타내었다. 농산물의 산술평균거칠기 (R_a) 값은 세 가지 병원균의 저감화 정도와 접촉각 보다는 작은 부적관계를 나타내었고, 기구 표면의 산술평균거칠기의 경우 세 가지 병원균의 저감화 정도와 유의적인 상관관계를 나타내지 않았다 ($p > 0.05$). 이러한 결과를 통해 샘플 표면의 소수성이 거칠기 특성보다 이산화염소의 식중독 균 저감화에 있어 더 큰 영향을 주는 요인이라는 것을 알 수 있었다.

이산화염소 가스의 처리 온도가 이산화염소 가스의 용해도 및 그에 따른 식중독 균 저감화 효과에 미치는 영향에 대한 연구를 수행하였다. 농산물 (시금치, 토마토) 및 기구 표면 (스테인레스 스틸 No.4, 유리)에

세 가지 병원균을 접종한 후 20 ppmv 이산화염소 가스를 동일한 절대습도 조건하에 15° C 및 25° C 에서 최대 30 분간 처리하였다. 이산화염소 가스 처리 시간이 증가할수록 25° C 보다 15° C 에서 세 가지 병원균의 유의적으로 ($p < 0.05$) 높은 저감화가 나타났다. 이산화염소 가스 (20 ppmv)를 15° C 에서 30 분간 처리하였을 때 25° C 에서의 처리 시 보다 시금치, 토마토, 스테인레스 스틸 No.4 에서 각각 세 가지 식중독 균의 0.99~1.65, 1.05~1.50, 1.25~1.61 log 더 높은 저감화 나타났다. 유리 표면의 경우 25° C 에서 20 분간 처리 시 세 가지 식중독 균의 1.88~2.31 log 저감화가 나타난 것에 비해 15° C 에서 15 분간 처리 시 세 가지 식중독 균 모두 검출한계 (0.48 log CFU/cm^2) 이하로 저감화 되었다. 이산화염소 가스 처리 후 샘플 표면에서의 이산화염소 농도를 측정하여 보았을 때, 15° C 에서 처리 시 모든 샘플에서 25° C 보다 유의적으로 ($p < 0.05$) 높은 이산화염소가 검출되었다. 따라서 이산화염소 가스 처리 시 처리 온도가 이산화염소 가스의 샘플 표면에서의 용해도에 영향을 미치며 그에 따른 식중독 균의 저감화에 영향을 미치는 것을 알 수 있었다.

이산화염소 가스의 살균 효율 증진을 위해 자외선 (ultraviolet, UV) 조사와의 병행처리 연구를 수행하였다. 시금치의 경우 이산화염소 가스 및 자외선 병행 처리 시 처리 시간이 증가함에 따라 세 가지 식중독 균의

저감화에 있어 상가효과 (additive effect)가 나타났다. 토마토의 경우 10 ppmv 이산화염소 가스와 자외선을 15 분간 병행 처리 시 *E. coli* O157:H7 및 *S.* Typhimurium 의 저감화에 있어 상승효과 (synergistic effect)가 나타났으며, *L. monocytogenes* 의 경우 20 분 병행 처리 시 상승효과가 나타났다. 상승효과의 원인을 규명하기 위해 병행 처리 후 식중독 균으로부터 나온 자외선 흡수 물질 (UV-absorbing substances)의 양을 측정하고, 투과전자현미경 (transmission electron microscope) 이미지를 확인하였다. 그 결과 상승효과의 주된 원인은 식중독 균의 세포막 손상에 의한 막 투과성 변화인 것을 알 수 있었다. 이산화염소 가스 (10 ppmv) 및 자외선 병행 처리에 따른 품질 변화를 확인하였을 때, 7 일간의 저장기간 동안 대조군 대비 색과 텍스쳐에 있어 유의적인 변화가 관찰되지 않았다 ($p > 0.05$).

다른 허들 기술로, 이산화염소 가스 (5, 10 ppmv) 및 에어로졸 살균제 (80 ppm 과초산)의 병행 처리 연구를 수행하였다. 시금치의 경우 처리 시간이 증가함에 따라 병행 처리 시 세 가지 식중독 균의 저감화에 있어 상가효과가 나타났다. 시금치에 10 ppmv 이산화염소 가스를 20 분간 처리 하였을 때, *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* 에 있어 각각 3.39, 3.29, 3.36 log 저감화가 나타났으며, 80 ppm 에어로졸 과초산 처리시 각각 2.27, 1.89, 0.84 log 저감화가

나타났다. 이에 비해 이산화염소 가스 (10 ppmv) 및 에어로졸 과초산 (80 ppm)를 20 분간 병행 처리 시 *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* 에 있어 각각 5.36, 5.06, 4.06 log 저감화가 나타났다. 토마토의 경우도 저감화에 있어 비슷한 경향성을 보였으며 병행 처리 시간이 증가함에 따라 상가 효과가 나타났다. 이산화염소 가스 (10 ppmv) 및 에어로졸 과초산의 병행 처리에 따른 품질 변화를 확인하였을 때, 7 일간의 저장기간 동안 대조군 대비 색과 텍스쳐에 있어 유의적인 변화가 관찰되지 않았다 ($p > 0.05$).

다음으로 이산화염소 가스 및 건열 (dry heat)의 연속적 처리 (sequential treatment)를 통한 알팔파 및 무 종자 내 식중독 균 제어 연구를 수행하였다. *E. coli* O157:H7 및 *S.* Typhimurium 이 접종 된 알팔파 및 무 종자에 150 ppmv 이산화염소 가스를 1 시간 동안 처리한 후, 70° C 및 80° C 에서 5 시간 동안 건열 처리를 하였다. 알팔파 종자의 경우 80° C 의 건열을 5 시간 동안 단독 처리 하였을 때 *E. coli* O157:H7, *S.* Typhimurium 에서 각각 3.08, 3.23 log 저감화가 나타났으며, 150 ppmv 이산화염소 가스를 1 시간 동안 단독 처리 하였을 때 각각 1.22~1.45, 1.58~1.61 log 저감화가 나타났다. 150 ppmv 이산화염소 가스 1 시간 처리한 후 80° C 의 건열을 5 시간 동안 연속 처리 하였을 때, *E. coli* O157:H7, *S.* Typhimurium 에서 각각 5.32,

5.29 log 이상의 저감화가 나타났다. 무 종자의 경우 80° C 의 건열을 5 시간 동안 단독 처리 하였을 때 *E. coli* O157:H7, *S.* Typhimurium 에서 각각 2.49, 2.27 log 저감화가 나타났으며, 150 ppmv 이산화염소 가스 1 시간 처리한 후 80° C 의 건열을 5 시간 동안 연속 처리 하였을 때 각각 4.38, 4.11 log 저감화가 나타났다. 전반적으로 발아율에서는 대조군 대비 유의적인 차이가 나타나지 않았으며 ($p > 0.05$), 이산화염소 가스 및 80° C 의 건열을 5 시간 처리한 무 종자의 경우만 유의적으로 발아율이 감소하는 것을 확인하였다 ($p < 0.05$).

마지막으로 장비 사용 없이 현장에서의 이산화염소 가스 처리 적용을 위한 휴대용 서방성 이산화염소 가스 발생 조성물 개발 연구를 수행하였다. 아염소산나트륨 (sodium chlorite) 및 구연산 (citric acid)를 이용하여 이산화염소 가스를 발생시켰으며, 규조토 (diatomaceous earth)를 이용하여 이산화염소 가스의 서방성 방출을 유도하였다. 또한 염화칼슘 (calcium chloride)을 수화 촉진제로 사용하였다. 다양한 조성비로 혼합물을 제조하여 22±1 ° C, 50 및 90% 상대습도 조건 하에서 36 시간 동안 이산화염소 가스 발생량을 모니터링 하였다. 상대습도 조건이 이산화염소 가스의 발생 정도에 큰 영향을 주는 것을 알 수 있었으며, 규조토 및 염화칼슘의 첨가를 통해 이산화염소 가스 발생 속도 및 최대 농도를 조절할 수 있었다. 규조토 9 g 및 12

g 을 아염소산나트륨 (0.25 g) 및 구연산 (0.14 g)과 혼합하였을 때, 90% 상대습도 조건에서 각각 26± 1 ppmv, 18 ± 1 ppmv 의 이산화염소 가스 농도가 23 시간, 28 시간 동안 지속되는 것을 관찰하였다. 50% 상대습도 조건에서 아염소산나트륨 (0.25 g) 및 구연산 (0.14 g)에 0.5 g 및 0.35 g 의 규조토를 첨가한 후 각각 0.05 g 의 염화칼슘을 첨가해 주었을 때, 11 ± 1 ppmv, 16 ± 1 ppmv 의 이산화염소 가스가 26 시간, 24 시간 동안 지속되는 것을 관찰하였다. 휴대용 서방성 조성물을 이용하여 발생된 이산화염소 가스 (10, 20, 30 ppmv)를 50% 및 90% 상대습도 조건에서 *E. coli* O157:H7 및 *S.* Typhimurium 이 접종 된 시금치와 토마토에 처리하였다. 90% 상대습도 조건에서 30 ppmv 이산화염소 가스를 시금치에 처리하였을 때 *E. coli* O157:H7 및 *S.* Typhimurium 각각 6.16, 5.48 log 이상의 저감화가 나타났으며, 토마토에서는 각각 6.78, 6.34 log 이상의 저감화가 나타났다.

본 연구를 통해 도출된 결과는 이산화염소 가스 살균 기술의 실제적인 산업적용에 있어 그 효과를 극대화 시키는데 활용될 수 있을 것이며, 현재 사용되고 있는 살균 기술들의 대안으로 활용될 수 있을 것이다. 또한 휴대용 서방성 이산화염소 가스 발생 조성물의 경우 이산화염소 가스의 현장 적용성을 보다 높여 줄 수 있을 것이다.

주제어 : 이산화염소 가스, 식중독 균, 저감화, 상대습도, 표면특성, 온도, 자외선, 에어로졸라이제이션, 건열, 서방성 가스 발생

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